PATENT APPLICATION

FACTOR VII OR VIIA-LIKE MOLECULES

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FACTOR VII OR VIIA-LIKE MOLECULES

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CROSS-REFERENCES TO RELATED APPLICATIONS

The present application claims priority to and benefit of the following United States and international patent applications: Danish Patent Application Number PA 2000 00218, filed February 11, 2000; United States Provisional Patent Application Number 60/184,036, filed

15   February 22, 2000; and, United States Provisional Patent Application Number 60/241,916, filed October 18, 2000.

FILED OF THE INVENTION

The present invention relates to novel Factor VII (FVII) or Factor VIIa (FVIIa) polypeptide conjugates, to their preparation and use in therapy, in particular for the treatment of

20   a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components (or factors) that eventually gives rise to a fibrin clot. Generally, the blood components participating in what has been referred to as the coagulation "cascade" are

25   proenzymes or zymogens, i.e., enzymatically inactive proteins that are converted into an active form by the action of an activator. One of these coagulation factors is FVII.

FVII is a vitamin K-dependent plasma protein synthesized in the liver and secreted into the blood as a single-chain glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an

30   activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa complex)

5   is able to convert both factor IX and factor X into their activated forms, followed by reactions

leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad

Sci USA 1977; 74:5260-5264).

FVII undergoes post-translational modifications, including vitamin K-dependent

carboxylation resulting in ten γ-carboxyglutamic acid residues in the N-terminal region of the

10 molecule. Thus, residue number 6, 7, 14, 16, 19, 20, 25, 26, 29 and 35 shown in SEQ ID NO:1

are γ-carboxyglutamic acids residues in the Gla domain important for FVII activity. Other post-

translational modifications include sugar moiety attachment at two naturally occurring N-

glycosylation sites at position 145 and 322, respectively, and at two naturally occurring O-

glycosylation sites at position 52 and 60, respectively.

15 The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at

q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans

12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation

and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant

proteins, with exons 1a and 1b encoding for signal sequence; exon 2 the propeptide and Gla

20 domain; exon 3 a short hydrophobic region; exons 4 and 5 the epidermal growth factor-like

domains; and exon 6 through 8 the serine protease catalytic domain (Yoshitake et al.,

Biochemistry 1985; 24: 3736-3750).

Reports exist on experimental three-dimensional structures of hFVIIa (Pike et al.,

PNAS. U.S.A., 1999; 96:8925-30 and Kemball-Cook et al., J.Struct.Biol, 1999; 127:213-223), of

25 hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods (Banner et

al., Nature, 1996; 380:41 and Zhang et al., J.Mol.Biol, 1999; 285: 2089), and of smaller

fragments of hFVII (Muranyi et al., Biochemistry, 1998; 37:10605 and Kao et al., Biochemistry,

1999; 38:7097).

Relatively few protein-engineered variants of FVII have been reported (Dickinson

30 & Ruf, J Bio Chem, 1997;272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-

8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999;

38:1957-1966).

Reports exist on expression of FVII in BHK or other mammalian cells

(WO92/15686, WO91/11514 and WO88/10295) and co-expression of FVII and kex2

35 endoprotease in eukaryotic cells (WO 00/28065).

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5   Commercial preparations of human recombinant FVIIa are sold as NovoSeven®.
NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients.
NovoSeven® is the only rFVIIa for effective and reliable treatment of bleeding episodes
available on the market.

An inactive form of FVII in which arginine 152 and/or isoleucine 153 is/are
10   modified has been reported in WO91/1154. These amino acids are located at the activation site.
WO 96/12800 describes inactivation of FVIIa by a serine proteinase inhibitor; inactivation by
carbamylation of FVIIa at the α-amino acid group I153 has been described by Petersen et al.,
Eur J Biochem, 1999;261:124-129. The inactivated form is capable of competing with wild-type
FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of
15   FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as
patients with sepsis, in risk of myocardial infarction or of thrombotic stroke.

A circulating rFVIIa half-life of 2.3 hours was reported in "Summary Basis for
Approval for NovoSeven®," FDA reference number 96-0597. Relatively high doses and
frequent administration are necessary to reach and sustain the desired therapeutic or prophylactic
20   effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent
intravenous administrations imposes restrictions on the patient's way of living.

Another problem in current rFVIIa treatment is the relative instability of the
molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for
obtaining a preparation in solution as opposed to a lyophilised product. The advantage of
25   obtaining a stable soluble preparation lies in easier handling for the patient, and, in the case of
emergencies, quicker action, which potentially can become life saving. Attempts to prevent
proteolytic degradation by site directed mutagenesis at major proteolytic sites have been
disclosed in WO88/10295.

A molecule with a longer circulation half-life would decrease the number of
30   necessary administrations. Given the association of current FVIIa product with frequent
injections, and the potential for obtaining more optimal therapeutic FVIIa levels with
concomitant enhanced therapeutic effect, there is a clear need for improved FVII or FVIIa-like
molecules.

5   One way to increase the circulation half-life of a protein is to ensure that renal clearance

of the protein is reduced. This can be achieved by conjugating the protein to a chemical moiety,

which is capable of conferring reduced renal clearance to the protein.

Furthermore, attachment of a chemical moiety to the protein or substitution of amino

acids exposed to proteolysis can effectively block a proteolytic enzyme from contact leading to

10   proteolytic degradation of the protein. Polyethylene glycol (PEG) is one such chemical moiety

that has been used in the preparation of therapeutic protein products.

WO98/32466 suggests that FVII, among many other proteins, can be PEGylated but

does not provide any further information in this respect.

SUMMARY OF THE INVENTION

15   This application discloses improved FVII and FVIIa molecules, in particular

recombinant hFVII and hFVIIa molecules, providing one or more of the aforementioned desired

benefits. Thus, the conjugate of the present invention has one or more improved properties as

compared to commercially available rFVIIa, including increased functional *in vivo* half-life

and/or increased plasma half-life, and/or increased bioavailability and/or reduced sensitivity to

20   proteolytic degradation. Consequently, medical treatment with a conjugate of the invention

offers a number of advantages over the currently available rFVIIa compound, such as longer

duration between injections.

Accordingly, in a first aspect, the invention relates to a conjugate comprising at

least one non-polypeptide moiety covalently attached to a polypeptide, wherein the amino acid

25   sequence of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1

in that at least one amino acid residue comprising an attachment group for said non-polypeptide

moiety has been introduced or removed.

In another aspect, the invention relates to a polypeptide, wherein the amino acid

sequence of the polypeptide differs from that of wild-type FVII or hFVIIa shown in SEQ ID

30   NO:1 in that at least one amino acid residue comprising an attachment group for a non-

polypeptide moiety has been introduced or removed. Such novel FVII polypeptides are

contemplated to be useful as such for therapeutic, diagnostic or other purposes, but find

particular interest as intermediate products for the preparation of a conjugate of the invention.

In further aspects, the invention relates to: a nucleotide sequence encoding the

35   polypeptide of the invention or the polypeptide part of the conjugate of the invention; an

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5   expression vector harbouring the nucleotide sequence of the invention; a host cell comprising the

nucleotide sequence of the invention or the expression vector of the invention.

In still further aspects, the invention relates to pharmaceutical compositions

comprising the conjugate of the invention as well as to methods for preparing and using such

conjugates.

10   **DETAILED DISCUSSION**

DEFINITIONS

In the context of the present application and invention the following definitions

apply:

The term "conjugate" (or interchangeably "conjugated polypeptide") is intended

15   to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the

covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such

as polymer molecules, lipophilic compounds, sugar moieties or organic derivatizing agents.

Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e., soluble in

physiological fluids such as blood. Examples of conjugated polypeptides of the invention include

20   glycosylated and/or PEGylated polypeptides.

The term "covalent attachment" means that the polypeptide and the non-

polypeptide moiety are either directly covalently joined to one another, or else are indirectly

covalently joined to one another through an intervening moiety or moieties, such as a bridge,

spacer, or linkage moiety or moieties.

25   The term "non-conjugated polypeptide" can be used about the polypeptide part of

the conjugate.

When used herein, the term "non-polypeptide moiety" means a molecule that is

capable of conjugating to an attachment group of the polypeptide of the invention. Preferred

examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds,

30   or organic derivatizing agents. When used in the context of a conjugate of the invention it will be

understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugate

through an attachment group of the polypeptide. As explained above, the non-polypeptide moiety

can be directly covalently joined to the attachment group or it can be indirectly covalently joined

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5   to the attachment group through an intervening moiety or moieties, such as a bridge, spacer, or

linkage moiety or moieties.

The "polymer molecule" is a molecule formed by covalent linkage of two or more

monomers, wherein none of the monomers is an amino acid residue, except where the polymer is

human albumin or another abundant plasma protein. The term "polymer" can be used

10   interchangeably with the term "polymer molecule." The term is intended to cover carbohydrate

molecules attached by *in vitro* glycosylation, i.e., a synthetic glycosylation performed *in vitro*

normally involving covalently linking a carbohydrate molecule to an attachment group of the

polypeptide, optionally using a cross-linking agent.

Carbohydrate molecules attached by *in vivo* glycosylation, such as N- or O-

15   glycosylation (as further described below) are referred to herein as a "sugar moiety." Except

where the number of non-polypeptide moieties, such as polymer molecule(s) or sugar moieties in

the conjugate is expressly indicated every reference to "a non-polypeptide moiety" contained in a

conjugate or otherwise used in the present invention shall be a reference to one or more non-

polypeptide moieties, such as polymer molecule(s) or sugar moieties.

20   The term "attachment group" is intended to indicate a functional group of the

polypeptide, in particular of an amino acid residue thereof or a carbohydrate moiety, capable of

attaching a non-polypeptide moiety such as a polymer molecule, a lipophilic molecule, a sugar

moiety or an organic derivatizing agent. Useful attachment groups and their matching non-

polypeptide moieties are apparent from the table below.

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Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/- Activated PEG	Reference
-NH$_2$	N-terminal, Lys	Polymer, e.g., PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)

-COOH	C-terminal, Asp, Glu	Polymer, e.g., PEG, with ester or amide group Carbohydrate moiety	mPEG-Hz *In vitro* coupling	Shearwater Inc.
-SH	Cys	Polymer, e.g., PEG, with disulfide, maleimide or vinyl sulfone group Carbohydrate moiety	PEG-vinylsul-phone PEG-maleimide *In vitro* coupling	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-OH	Ser, Thr, Lys, OH-	Sugar moiety PEG with ester, ether, carbamate, carbonate	*In vivo* O-linked glycosylation	
-CONH$_2$	Asn as part of an N-glycosyla-tion site	Sugar moiety Polymer, e.g., PEG	*In vivo* N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	*In vitro* coupling	
-CONH$_2$	Gln	Carbohydrate moiety	*In vitro* coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759-65

Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g., PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179:301, WO 92/16555, WO 00/23114
Guanidino	Arg	Carbohydrate moiety	*In vitro* coupling	Lundblad and Noyes, Chimical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Carbohydrate moiety	*In vitro* coupling	As for guanidine

For *in vivo* N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting a N-glycosylation site (with the sequence N-X-S/T/C, wherein X is any amino acid residue except proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present.

Accordingly, when the non-polypeptide moiety is a sugar moiety and the conjugation is to be achieved by N-glycosylation, the term "amino acid residue comprising an attachment group for the non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide of interest is to be understood as meaning that one or more amino acid residues constituting an N-glycosylation site are to be altered in such a manner that either a functional N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

In the present application, amino acid names and atom names (e.g., CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) (www.pdb.org) based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and

5 Peptides (residue names, atom names, etc.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985)).

The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H),

10 isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

The terminology used for identifying amino acid positions is illustrated as follows: G124 indicates that position 124 is occupied by a glycine residue in the amino acid

15 sequence shown in SEQ ID NO:1. G124R indicates that the glycine residue of position 124 has been substituted with an arginine residue. Alternative substitutions are indicated with a "/," e.g., K32D/E means an amino acid sequence in which lysine in position 32 is substituted with either aspartic acid or glutamic acid. Multiple substitutions are indicated with a "+," e.g., K143N+N145S/T means an amino acid sequence which comprises a substitution of the lysine

20 residue in position 143 with an asparagine residue and a substitution of the asparagine residue in position 145 with a serine or a threonine residue. The insertion of an additional amino acid residue, such as insertion of an alanine residue after G124 is indicated by G124GA. A deletion of an amino acid residue is indicated by an asterix. For example, deletion of a glycine in position 124 is indicated by G124*. Unless otherwise indicated, the numbering of amino acid residues

25 made herein is made relative to the amino acid sequence of wild-type FVII/FVIIa shown in SEQ ID NO:1.

The term "differs from" as used in connection with specific mutations is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the removal and/or introduction of amino acid residues comprising an

30 attachment group for the non-polypeptide moiety the FVII or FVIIa polypeptide can comprise other substitutions that are not related to introduction and/or removal of such amino acid residues. Thus, in addition to the amino acid alterations disclosed herein aimed at removing and/or introducing attachment sites for the non-polypeptide moiety, it will be understood that the amino acid sequence of the polypeptide of the invention can, if desired, contain other alterations

35 that need not be related to introduction or removal of attachment sites, i.e., other substitutions,

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5   insertions or deletions. These can, for example, include truncation of the N- and/or C-terminus

by one or more amino acid residues, or addition of one or more extra residues at the N- and/or C-

terminus, e.g., addition of a methionine residue at the N-terminus as well as "conservative amino

acid substitutions," i.e., substitutions performed within groups of amino acids with similar

characteristics, e.g., small amino acids, acidic amino acids, polar amino acids, basic amino acids,

10   hydrophobic amino acids and aromatic amino acids.

Preferred substitutions in the present invention can in particular be selected from

the conservative substitution groups listed in the table below.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

15   The terms "mutation" and "substitution" are used interchangeably herein.

The term "nucleotide sequence" is intended to indicate a consecutive stretch of

two or more nucleotide molecules. The nucleotide sequence can be of genomic, cDNA, RNA,

semisynthetic, synthetic origin, or any combinations thereof.

The term "polymerase chain reaction" or "PCR" generally refers to a method for

20   amplification of a desired nucleotide sequence *in vitro*, as described, for example, in US

4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis,

using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

"Cell," "host cell," "cell line" and "cell culture" are used interchangeably herein

and all such terms should be understood to include progeny resulting from growth or culturing of

25   a cell. "Transformation" and "transfection" are used interchangeably to refer to the process of

introducing DNA into a cell.

"Operably linked" refers to the covalent joining of two or more nucleotide

sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one

another such that the normal function of the sequences can be performed. For example, the

5 nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence coding for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked"

10 means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

The term "introduce" refers to introduction of an amino acid residue comprising

15 an attachment group for a non-polypeptide moiety, in particular by substitution of an existing amino acid residue, or alternatively by insertion of an additional amino acid residue.

The term "remove" refers to removal of an amino acid residue comprising an attachment group for a non-polypeptide moiety, in particular by substitution of the amino acid residue to be removed by another amino acid residue, or alternatively by deletion (without

20 substitution) of the amino acid residue to be removed

The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form.

The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two-chain form, wherein the peptide bond between R152 and I153 of the single-

25 chain form has been cleaved. When the amino acid sequence of SEQ ID NO;1 is used herein to describe the amino acid sequence of FVIIa it will be understood to that one of the chains comprises amino acid residues 1-152, the other chain amino acid residues 153-406.

The terms "rFVII" and "rFVIIa" refer to FVII and FVIIa molecules produced by recombinant techniques, respectively.

30 The terms "hFVII" and "hFVIIa" refer to wild-type human FVII and FVIIa, respectively.

The term "catalytic site " is used to mean the catalytic triad consisting of S344, D242 and H193 of the FVII polypeptide.

The term "active FVIIa," "active FVIIa polypeptide," "active FVIIa conjugate" or

35 "active conjugate" is used to mean a FVIIa polypeptide or conjugate that possesses at least 10%

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5 of the catalytic activity of wild-type hFVIIa. Catalytic acitivity, as used herein, can suitably be determined in the assay described in the section entitled *Method of measuring the catalytic activity* or in the assay entitled *Method of measuring low levels of catalytic activity* (see the Materials and Methods section herein). Preferably, an active conjugate has at least 15%, such as at least 20%, e.g., at least 25%, more preferably at least 30%, such as at least 40%, most

10 preferably at least 50%, e.g., at least 60% of the catalytic acitivity of wild-type hFVIIa, when tested in the assays described above.

Preferably, an active conjugate is able to bind to tissue factor and further activate plasma factor X and/or IX. Thus, in a preferred embodiment, the active FVIIa polypeptide or a conjugate thereof has a clotting activity of at least 25% as compared to wild type FVIIa, such as

15 a clotting activity of at least 50% as compared to wild type FVIIa, e.g., a clotting activity of at least 75% as compared to wild type FVII. Thus, the clotting activity of the active FVIIa polypeptide or conjugate thereof is preferably in the range of 25-200% as compared to the wild type FVIIa. In particular, it is preferred that the FVIIa polypeptide or conjugate has a clotting activity in the range of 30–150% as compared to wild type FVIIa, such as a clotting activity in

20 the range of 30-100% as compared to wild type FVIIa. The clotting activity can be determined by any method known in the art as further discussed in the Materials and Methods section hereinafter. It is particularly preferred, however, that the clotting activity is determined in accordance with the method described in the section entitled *"Method of measuring the clotting activity"* (see the Materials and Method section).

25 The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response can be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally, reduced antibody reactivity will be an indication of reduced immunogenicity. The reduced

30 immunogenicity can be determined by use of any suitable method known in the art, e.g., *in vivo* or *in vitro*.

The term "inactive FVIIa," "inactive FVIIa polypeptide," "inactive FVIIa conjugate" or "inactive conjugate" is used to mean a FVIIa polypeptide or conjugate that possesses less than 10% of the catalytic activity of wild-type hFVIIa. Catalytic acitivity, as used

35 herein, can suitably be determined in the assay described in the section entitled *Method of*

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5   *measuring the catalytic activity* or in the assay entitled *Method of measuring low levels of*

catalytic activity (see the Materials and Methods section herein). Preferably, an inactive

conjugate has less than 8%, such as less than 6%, e.g., less than 5%, more preferably less than

4%, such as less than 3%, most preferably less than 2%, e.g., less than 1% of the catalytic

acitivity of wild-type hFVIIa, when tested in the assays described above.

10   Typically, an inactive conjugate has significantly reduced *in vitro* or *in vivo*

clotting activity as compared to wild-type hFVIIa. The inactive FVII or FVIIa polypeptide or

conjugate can be capable of competing with wild-type FVII or FVIIa for binding tissue factor,

thereby inhibiting clotting activity. Preferably, the inactive FVII or FVIIa polypeptide or

conjugate has less than 1% clotting activity compared to wild-type hFVII or hFVIIa. More

15   preferably the inactive FVII or FVIIa polypeptide or conjugate has less than 0.05% clotting

activity compared to wild type hFVII or hFVIIa. Most preferably the inactive FVII or FVIIa

polypeptide or conjugate has less than 0.01% clotting activity as compared to wild type hFVII or

hFVIIa. In a similar way as described above, the clotting activity can be determined by any

method known in the art as further discussed in the Materials and Method section hereinafter, but

20   is preferably determined in accordance with the method described in the section entitled

"Method of measuring the clotting activity."

The term "functional *in vivo* half-life" is used in its normal meaning, i.e., the time

at which 50% of the biological activity of the polypeptide or conjugate is still present in the

body/target organ, or the time at which the activity of the polypeptide or conjugate is 50% of the

25   initial value. As an alternative to determining functional *in vivo* half-life, "serum half-life" can be

determined, i.e., the time at which 50% of the polypeptide or conjugate molecules circulate in the

plasma or bloodstream prior to being cleared. Determination of serum half-life is often more

simple than determining the functional *in vivo* half-life and the magnitude of serum half-life is

usually a good indication of the magnitude of functional *in vivo* half-life. Alternatively terms to

30   serum half-life include "plasma half-life," "circulating half-life," "serum clearance," "plasma

clearance" and "clearance half-life." The polypeptide or conjugate is cleared by the action of one

or more of the reticuloendothelial systems (RES), kidney, spleen or liver, by tissue factor, SEC

receptor or other receptor mediated elimination, or by specific or unspecific proteolysis.

Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge,

35   attached carbohydrate chains, and the presence of cellular receptors for the protein. The

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5   functionality to be retained is normally selected from procoagulant, proteolytic or receptor binding activity. The functional *in vivo* half-life and the serum half-life can be determined by any suitable method known in the art as further discussed in the Materials Methods section below.

The term "increased" as used about the functional *in vivo* half-life or plasma half-life is used to indicate that the relevant half-life of the conjugate or polypeptide is statistically

10   significantly increased relative to that of a reference molecule, such as a non-conjugated rFVIIa (e.g., NovoSeven®) as determined under comparable conditions. For instance, the relevant half-life can increased by at least about 25%, such as by at least about 50%, e.g., by at least about 100%, 150%, 200%, 250%, 300%, 500% or 1000%.

The term "renal clearance" is used in its normal meaning to indicate any clearance

15   taking place by the kidneys, e.g., by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the conjugate, including size (diameter), hydrodynamic volume, symmetry, shape/rigidity, and charge. Normally, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. Renal clearance can be established by any suitable assay, e.g., an established *in vivo* assay. Typically, renal

20   clearance is determined by administering a labelled (e.g., radiolabelled or fluorescence labelled) polypeptide conjugate to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g., the corresponding non-conjugated polypeptide, a non-conjugated corresponding wild-type polypeptide or another conjugated polypeptide (such as a conjugated polypeptide not according

25   to the invention), under comparable conditions. Preferably, the renal clearance rate of the conjugate is reduced by at least 50%, preferably by at least 75%, and most preferably by at least 90% compared to a relevant reference polypeptide.

The ability of the conjugates of the invention to exhibit a reduced sensitivity to proteolytic degradation is of utmost importance; Compositions comprising degradation products

30   will typically have less specific activity as compared to compositions in which none or only a minor part of the conjugate has been degraded. Furthermore, a content of non-physiological degradation products in the composition to be administered can trigger the immune system of the patient.

The term "reduced sensitivity to proteolytic degradation" is primarily intended to

35   mean that the conjugate has reduced sensitivity to proteolytic degradation in comparison to non-

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5   conjugated wild type FVIIa as determined under comparable conditions. Preferably, the
proteolytic degradation is reduced by at least 10%, such as at least 25% (e.g., by 10-25%), more
preferably by at least 35%, such as at least 50%, (e.g., by 10-50%, such as 25-50%) even more
preferably by at least 60%, such as by at least 75% or even at least 90%. Most preferably, the
proteolytic degradation is reduced by 100%. Thus, preferably the conjugate of the invention is
10   subjected to proteolytic degradation to a lesser extent than wild-type FVIIa, i.e., compared to
non-conjugated wild type FVIIa the proteolytic degradation of the conjugate of the invention is
preferably reduced by 10-100%, such as by 25-100%, more preferably by 50-100%, and most
preferably by 75-100%.

The present inventors have developed a suitable preliminary *in vitro* test, which
15   can be employed in the assessment of whether such conjugates possess reduced sensitivity to
proteolytic cleavage (reduced autoproteolysis). Thus, in a preferred embodiment, of the
invention, the conjugate of the invention has a reduced sensitivity to proteolytic degradation (as
defined above) as compared to wild type FVIIa when determined by the method described in the
section entitled *"Measurement of reduced sensitivity to proteolytic degradation,"* when
20   determined by the method described in the section entitled *Method of measuring the catalytic
activity* or when determined by the method described in the section entitled *Method of measuring
low levels of catalytic activity* (see the Materials and Methods section herein).

The term "parent FVII" or "parent polypeptide" is intended to indicate the
molecule to be modified in accordance with the present invention. A typical parent FVII is the
25   hFVII or hFVIIa (including rFVIIa (NovoSeven®)) with the amino acid sequence shown in SEQ
ID NO:1.

A "variant" is a polypeptide, which differs in one or more amino acid residues
from a parent polypeptide, normally in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid
residues.

30   <u>Conjugate of the invention</u>
The conjugates of the invention are the result of a generally new strategy for
developing improved FVII or FVIIa molecules. More specifically, by removing and/or
introducing an amino acid residue comprising an attachment group for the non-polypeptide
moiety it is possible to specifically adapt the polypeptide so as to make the molecule more
35   susceptible to conjugation to the non-polypeptide moiety of choice, to optimize the conjugation

15

5   pattern (e.g., to ensure an optimal distribution and number of non-polypeptide moieties on the surface of the FVII or FVIIa molecule and to ensure that only the attachment groups intended to be conjugated is present in the molecule) and thereby obtain a new conjugate molecule, which has or has not FVII activity and in addition one or more improved properties as compared to FVII and FVIIa molecules available today. For instance, when the total number of amino acid

10   residues comprising an attachment group for the non-polypeptide of choice is increased or decreased to an optimized level, the renal clearance of the conjugate is typically significantly reduced due to the altered shape, size and/or charge of the molecule achieved by the conjugation.

In preferred embodiments of the present invention more than one amino acid residue of the FVII or FVIIa polypeptide is altered, e.g., the alteration embraces removal as well

15   as introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice. In addition to the removal and/or introduction of amino acid residues the polypeptide can comprise other substitutions or glycosylations that are not related to introduction and/or removal of amino acid residues comprising an attachment group for the non-polypeptide moiety. Also, the polypeptide can be attached, e.g., to a serine proteinase inhibitor to inhibit the

20   catalytic site of the polypeptide.

The amino acid residue comprising an attachment group for a non-polypeptide moiety, either it be removed or introduced, is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide and the non-polypeptide moiety is to be achieved. For

25   instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or polyalkylene oxide derived molecule amino acid residues comprising an attachment group can be selected from the group consisting of lysine, cysteine, aspartic acid, glutamic acid, histidine, and tyrosine, preferably cysteine and lysine, in particular lysine.

Whenever an attachment group for a non-polypeptide moiety is to be introduced

30   into or removed from the FVII or FVIIa polypeptide in accordance with the present invention, the position of the polypeptide to be modified is preferably located at the surface of the poly-peptide, and more preferably occupied by an amino acid residue which has more than 25% of its side chain exposed to the solvent, preferably more than 50% of its side chain exposed to the solvent. Such positions have been identified on the basis of an analysis of a 3D structure of the

35   human FVII or FVIIa molecule as described in the Materials and Methods section herein.

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5   Furthermore, the position is preferably selected from a part of the FVII molecule that is located
      outside a tissue factor binding site region and/or an active site region. These regions are
      identified in the Materials and Methods section hereinafter. It should be emphasized, however,
      that in certain situations, e.g., in case an inactivated conjugate is desired, it can be advantageous
      to perform modifications in or close to such regions. For example, it is contemplated that one or
10   more attachment groups for the non-polypeptide moieties, such as attachment groups for *in vivo*
      N-glycosylation sites, can advantageously be inserted in the active site region or at the ridge of
      the active site binding cleft of the FVII molecule. The active site region and the ridge of the
      active site binding cleft are defined in the Materials and Methods section herein and is
      constituted by the following residues:

15   I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181,
      V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229,
      I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244,
      L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328,
      D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360,
20   I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386,
      L387, L400 and F405 (active site region); and N173, A175, K199, N200, N203, D289, R290,
      G291, A292, P321 and T370 (the ridge of the active site binding cleft).

                In order to determine an optimal distribution of attachment groups, the distance
      between amino acid residues located at the surface of the FVII or FVIIa molecule is calculated
25   on the basis of a 3D structure of the polypeptide. More specifically, the distance between the
      CB's of the amino acid residues comprising such attachment groups, or the distance between the
      functional group (NZ for lysine, CG for aspartic acid, CD for glutamic acid, SG for cysteine) of
      one and the CB of another amino acid residue comprising an attachment group are determined.
      In case of glycine, CA is used instead of CB. In the FVII or FVIIa polypeptide part of a
30   conjugate of the invention, any of said distances is preferably more than 8 Å, in particular more
      than 10Å in order to avoid or reduce heterogeneous conjugation.

                In case of removal of an attachment group, the relevant amino acid residue
      comprising such group and occupying a position as defined above is preferably substituted with a
      different amino acid residue that does not comprise an attachment group for the non-polypeptide
35   moiety in question. Normally, the amino acid residue to be removed is one to which conjugation

5   is disadvantageous, e.g., an amino acid residue located at or near a functional site of the

polypeptide (since conjugation at such a site can result in inactivation or reduced FVII or FVIIa

activity of the resulting conjugate due to impaired receptor recognition). In the present context

the term "functional site" is intended to indicate one or more amino acid residues which is/are

essential for or otherwise involved in the function or performance of FVII or FVIIa. Such amino

10   acid residues are a part of the functional site. The functional site can be determined by methods

known in the art and is preferably identified by analysis of a structure of the FVIIa-tissue factor

complex (See Banner et al., Nature 1996; 380:41-46).

In case of introduction of an attachment group, an amino acid residue comprising

such group is introduced into the position, preferably by substitution of the amino acid residue

15   occupying such position.

The exact number of attachment groups present and available for conjugation in

the FVII or FVIIa polypeptide is dependent on the effect desired to be achieved by the

conjugation. The effect to be obtained is, e.g., dependent on the nature and degree of conjugation

(e.g., the identity of the non-polypeptide moiety, the number of non-polypeptide moieties

20   desirable or possible to conjugate to the polypeptide, where they should be conjugated or where

conjugation should be avoided, etc.).

Functional *in vivo* half-life is *i.a.* dependent on the molecular weight of the

conjugate, and the number of attachment groups needed for providing increased half-life thus

depends on the molecular weight of the non-polypeptide moiety in question. In one embodiment,

25   the conjugate of the invention has a molecular weight of at least 67 kDa, in particular at least 70

kDa, e.g., as measured by SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970),

p680-85. FVII has a molecular weight of about 53 kDa, and therefore additional 10-20kDa is

required to obtain the desired effect. This can, e.g., be provided by conjugating 2-4 10kDa PEG

molecules or as otherwise described herein.

30   In order to avoid too much disruption of the structure and function of the parent

molecule the polypeptide part of the conjugate will typically have an amino acid sequence

having more than 90% identity with SEQ ID NO:1, preferably more than 95%, such as more than

96%. In particular, the polypeptide part of the conjugate will typically have an amino acid

sequence having more than 97% identity with SEQ ID NO:1, such as more than 98%, more than

35   99%, more than 99.25%, more than 99.25% or more than 99.5%.

5   Amino acid sequence homology/identity is conveniently determined from aligned sequences, using e.g., the ClustalW program, version 1.8, June 1999, using default parameters (Thompson et al., 1994, ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Research, 22: 4673-4680) or from the PFAM families database version 4.0

10   (http://pfam.wustl.edu/) (*Nucleic Acids Res.* 1999 Jan 1; 27(1):260-2) by use of GENEDOC version 2.5 (Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. 1997 GeneDoc: Analysis and Visualization of Genetic Variation, EMBNEW.NEWS 4:14; Nicholas, K.B. and Nicholas H.B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

          Stated differently, the total number of amino acid residues to be altered in

15   accordance with the present invention (as compared to the amino acid sequence shown in SEQ ID NO:1) will typically not exceed 15. Preferably, the FVII or FVIIa polypeptide part of the conjugate of the invention or the polypeptide of the invention comprises an amino acid sequence differing in 1-15 amino acid residues from the amino acid sequence shown in SEQ ID NO:1, typically in 1-10 or in 2-10 amino acid residues, e.g., in 1-8 or in 2-8 amino acid residues, such

20   as in 3-7 or in 4-6 amino acid residues from the amino acid sequence shown in SEQ ID NO:1. Thus, normally the polypeptide part of the conjugate or the polypeptide of the invention comprises an amino acid sequence which differs from the amino acid sequence shown in SEQ ID NO:1 in at the most 15 amino acid residues (such as 15 amino acid residues), in at the most 14 amino acid residues (such as 14 amino acid residues), in at the most 13 amino acid residues (e.g.,

25   13 amino acid residues), in at the most 12 amino acid residues (such as 12 amino acid residues), in at the most 11 amino acid residues (such as 11 amino acid residues), in at the most 10 amino acid residues (e.g., 10 amino acid residues), in at the most 9 amino acid residues (such as 9 amino acid residues), in at the most 8 amino acid residues (such as 8 amino acid residues), in at the most 7 amino acid residues (such as 7 amino acid residues), in at the most 6 amino acid

30   residues (such as 6 amino acid residues), in at the most 5 amino acid residues (such as 5 amino acid residues), in at the most 4 amino acid residues (such as 4 amino acid residues), in at the most 3 amino acid residues (such as 3 amino acid residues) or in at the most 2 amino acid residues (such as 2 amino acid residues).

5   Analogously, the conjugate of the invention typically contains, e.g., 1-15 non-
polypeptide moieties, typically 1-10 non-polypeptide moieties or 2-10 non-polypeptide moieties,
such as 1-8 or 2-8 non-polypeptide moieties, e.g., 1-6, 1-4, 3-7 or 4-6 non-polypeptide moieties.

           Preferably, the conjugate of the invention has one or more of the following
improved properties: Increased functional *in vivo* half-life, increased plasma half-life, reduced
10   renal clearance and reduced sensitivity to proteolytic degradation as compared to rFVIIa
(e.g.NovoSeven®).

           It is known from, *inter alia,* WO 88/10295 that proteolytic degradation of
FVII/FVIIa primarily takes place at various proteolytic sites in the molecule, namely at the
positions K32, K38, I42, Y44, K143, R290, R315, K341, R392, R396 and R402 (or rather
15   between the positions K32-D33, K38-L39, I42-S43, Y44-S45, K143-R144, R290-G291, R315-
K316, K341-G342, R392-S393, R396-P397 and R402-A403). Thus, one preferred strategy for
increasing e.g., the functional *in vivo* half-life, the increased plasma half-life or for reducing the
sensitivity to proteolytic degradation is to modify the parent polypeptide at and/or around one or
more of these proteolytic degradation sites, e.g., by introducing non-polypeptide moieties at
20   and/or around these sites. Thus, in a preferred embodiment, of the invention, an attachment
group has been introduced in one or more of the above-mentioned identified positions or at
position –4, -3, -2, -1, 1, 2, 3, 4, preferably position –2, -1, 1, 2, such as position –1, 1, relative to
the position directly involved in proteolytic degradation.

           More specifically, it is preferred that a non-naturally occurring *in vivo*
25   glycosylation site, in particular a non-naturally occurring *in vivo* N-glycosylation site (see further
below) has been introduced in positions selected from the group consisitng of 28-48, 139-147,
286-294, 311-319, 338-345, and 388-406. In particular, it is preferred that the *in vivo*
glycosylation site, such as an *in vivo* N-glycosylation site (see further below) has been
introduced in positions selected from the group consisiting of 30-34, 36-46, 141-144, 288-292,
30   313-317, 341-343, 390-398 and 400-404. More preferably, the *in vivo* glycosylation site, such as
an *in vivo* N-glycosylation site (see further below) has been introduced in positions selected from
the group consisiting of 31-33 (e.g., positions 31, 32 or 33), 37-39 (e.g., positions 37, 38 or 39),
41-46 (e.g., positions 41, 42, 43, 44, 45 or 46), 142-144 (e.g., positions 142, 143 or 144), 289-
291 (e.g., positions 289, 290 or 291), 314-316, (e.g., positions 314, 315 or 316), 341-342 (e.g.,
35   positions 341, 342 or 343), 391-393 (e.g., positions 391, 392 or 393), 395-397 (e.g., positions

20

5   395, 396 or 397) and 401-403 (e.g., positions 401, 402 or 403). The introduction is preferably

performed by susbstitution.

Conjugate of the invention, wherein the non-polypeptide moiety is a molecule that
has lysine as an attachment group

In another embodiment of the invention, the non-polypeptide moiety has lysine as

10   an attachment group, i.e., the conjugate of the invention is one which comprises at least one non-

polypeptide moiety covalently attached to a polypeptide, wherein the amino acid sequence of the

polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at least

one lysine residue has been introduced or removed.

FVII/FVIIa contains 17 lysine residues. Three lysine residues (K18, K62 and

15   K85) are located in the tissue factor binding domain and two lysine residues (K197 and K341)

are located in the active site region.

Due to the relative high amount of lysine residues in the parent polypeptide it is

envisaged that at least one lysine residue should preferably be removed, in particular by

substitution of a lysine residue with a non-lysine residue, in order to avoid excessive conjugation

20   to non-polypeptide moieties.

Thus, in one embodiment, the amino acid sequence of the FVII or FVIIa

polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one

lysine residue, such as 1-15 lysine residues, in particular 1-10, 1-6 or 2-4 lysine residues, has

been removed, preferably by substitution. For example, the lysine residue(s) to be removed,

25   preferably by substitution, is selected from the group consisting of K18, K32, K38, K62, K85,

K109, K137, K143, K148, K157, K161, K197, K199, K316, K337, K341, K389 and

combinations thereof. In particular, it is preferred to remove one or more lysine residues, which

constitute part of tissue factor binding site and/or the active site region, i.e., the residues K18,

K62, K85, K197, K341 or combinations thereof. The lysine residue can be substituted with any

30   other amino acid residue, but is preferably substituted with R, Q, N or H, more preferably R.

In another embodiment, the amino acid sequence of the FVII or FVIIa

polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one

lysine residue, such as 1-15 lysine residues, in particular 1-10, 1-6 or 2-4 lysine residues, has

been introduced, preferably by substitution. It will be understood that it is particularly preferred

35   that at least one of the lysines residues, which are introduced at a predetermined site in the parent

molecule, is combined with at least one of the above-mentioned removals of lysine residues.

21

5   Thus, in a preferred embodiment of the invention, the amino acid sequence of the FVII or FVIIa

polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one

lysine residue has been removed and at least one lysine residue has been introduced.

Examples of positions, wherein lysine residues can be introduced include, but is

not limited to, positions at or in the vicinity of the proteolytic degradation sites described above.

10   Thus, in a preferred embodiment, the substitution of a non-lysine residue with a lysine residue is

selected from the group consisting of of I42K, Y44K, L288K, D289K, R290K, G291K, A292K,

T293K, Q313K, S314K, R315K, V317K, L390K, M391K, R392K, S393K, E394K, P395K,

R396K, P397K, G398K, V399K, L400K, L401K, R402K, A403K, P404K, F405K and

combinations thereof, in particular selected from the group consisting of R290K, R315K,

15   R392K, R396K, R402K and combinations thereof.

While the non-polypeptide moiety of the conjugate according to this aspect of the

invention can be any molecule which, when using the given conjugation method has lysine as an

attachment group it is preferred that the non-polypeptide moiety is a polymer molecule. The

polymer molecule can be any of the molecules mentioned in the section entitled "Conjugation to

20   a polymer molecule," but is preferably selected from the group consisting of linear or branched

polyethylene glycol or another polyalkylene oxide. Examples of preferred polymer molecules

are, e.g., SS-PEG, NPC-PEG, aldehyde-PEG, mPEG-SPA, mPEG-SCM, mPEG-BTC from

Shearwater Polymers, Inc, SC-PEG from Enzon, Inc., tresylated mPEG as described in US

5,880,255, or oxycarbonyl-oxy-N-dicarboxyimide-PEG (US 5,122,614).

25   Normally, for conjugation to a lysine residue the non-polypeptide moiety has a

molecular weight of from about 5 to about 20 kDa, such as from about 5 to about 10 kDa, e.g.,

about 5 kDa or about 10 kDa

It will be understood that any of the amino acid changes, in particular

substitutions, specified in this section can be combined with any of the amino acid changes,

30   preferably substitutions specified in the other sections herein disclosing specific amino acid

modifications, including introduction and/or removal of glycosylation sites.

Conjugate of the invention wherein the non-polypeptide moiety is a molecule that
has cysteine as an attachment group

In a further embodiment of the invention, the non-polypeptide moiety has cysteine

35   as an attachment group, i.e., the conjugate of the invention is one which comprises at least one

non-polypeptide moiety covalently attached to a polypeptide, wherein the amino acid sequence

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5   of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at

least one cysteine residue has been introduced or removed.

FVII/FVIIa contains 24 cysteine residues and disulfide bridges are established

between the following cysteine residues: C17 and C22, C50 and C61, C55 and C70, C72 and

C81, C91 and C102, C98 and C112, C114 and C127, C135 and C262, C159 and C164, C178 and

10   C194, C310 and C329, and between C340 and C368.

In a further interesting embodiment, the amino acid sequence of the FVII or FVIIa

polypeptide differs from that shown in SEQ ID NO:1 in that at least one cysteine residue, such as

1-15 cysteine residues, in particular 1-10, 1-6 or 2-4 cysteine residues, has been introduced,

preferably by substitution.

15   Examples of positions, wherein cysteine residues can be introduced include, but is

not limited to, positions at or in the vicinity of the proteolytic degradation sites described above.

Thus, in an interesting embodiment of the invention, the lysine residue(s) to be

introduced, preferably by substitution, is selected from the group consisting of I30C, K32C,

D33C, A34C, T37C, K38C, W41C, Y44C, S45C, D46C, L141C, E142C, K143C, R144C,

20   L288C, D289C, R290C, G291C, A292C, S314C, R315C, K316C, V317C, L390C, M391C,

R392C, S393C, E394C, P395C, R396C, P397C, G398C, V399C, L401C, R402C, A403C,

P404C and combinations thereof, in particular selected from the group consisting of K32C,

Y44C, K143C, R290C, R315C, K341C, R392C, R396C, R402C and combinations thereof.

In a further embodiment of the invention, the cysteine residue(s) is/are introduced

25   into a position that in wildtype hFVII is occupied by a threonine or serine residue having at least

25% of its side chain exposed to the surface. For instance, in the FVII or FVIIa polypeptide a

cysteine residue is introduced, preferably by substitution, into at least one position selected from

the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, T83, S103, T106, T108, S111,

S119, S126, T128, T130, S147, T185, S214, S222, S232, T233, T238, T239, T255, T267, T293,

30   T307, S320, T324, S333, S336, T370 and S393. Even more preferably the cysteine residue is

introduced into at least one position of hFVII containing an S residue, the position being selected

from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, S103, S111, S119, S126,

S147, S214, S222, S232, S320, S333, S336 and S393.

In a further embodiment, the cysteine residue(s) is/are introduced into a position

35   that in wildtype hFVII is occupied by a threonine or serine residue having at least 50% of its side

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5 chain exposed to the surface. For instance, in the FVII or FVIIa polypeptide a cysteine residue is introduced, preferably by substitution, into at least one position selected from the group consisting of S23, S43, S52, S53, S60, S67, T106, T108, S111, S119, S147, S214, T238, T267 and T293, even more preferably a position selected from the group consisting of S23, S43, S52, S53, S60, S67, S111, S119, S147 and S214.

10 In a still further embodiment, a cysteine residue is introduced into at least one position selected from any of the above-mentioned positions, which is not located in an active site region. Preferably, the position is one occupied by a T or an S residue. As an example, the FVII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, T83, S103, T106, T108, S111,

15 S119, S126, T128, T130, S147, T185, S214, S222, T255, T267, T307, S320, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in particular selected from the group consisting of S12, S23, S43, S45, S52, S53, S60, S67, S103, S111, S119, S126, S147, S214, S222, S320, S333, S336 and S393 (occupied by S residue), and more preferably from the group consisting of S23, S43, S52, S53, S60, S67, T106, T108, S111, S119, S147, S214

20 and T267 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S43, S52, S53, S60, S67, S111, S119, S147 and S214 (occupied by an S residue).

In an even further embodiment, a cysteine residue is introduced into at least one position selected from any of the above lists, which is not located in a tissue factor binding site

25 region. Preferably, the position is one occupied by a T or an S residue. As an example, the FVII polypeptide comprises a cysteine residue introduced into at least one position selected from the group consisting of S12, S23, S45, S52, S53, S67, T83, S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, S232, T233, T238, T239, T255, T267, T293, S320, T324, S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in

30 particular selected from the group consisting of S12, S23, S45, S52, S53, S67, S103, S111, S119, S126, S147, S214, S222, S232, S320, S333, S336 and S393 (occupied by S residue), and more preferably from the group consisting of S23, S52, S53, S67, T106, T108, S111, S119, S147, S214, T238, T267 and T293 (having more than 50% of its side chain exposed to the surface), in particular from the group consisting of S23, S52, S53, S67, S111, S119, S147 and S214

35 (occupied by an S residue).

24

5   In a still further embodiment, a cysteine residue is introduced into at least one

position selected from any of the above lists, which is neither located in a tissue factor binding

site region nor in an active site region. Preferably, the position is one occupied by a T or an S

residue. As an example, the FVII polypeptide comprises a cysteine residue introduced into at

least one position selected from the group consisting of S12, S23, S45, S52, S53, S67, T83,

10   S103, T106, T108, S111, S119, S126, T128, T130, S147, T185, S214, S222, T255, T267, S320,

S333, S336, T370 and S393 (having more than 25% of its side chain exposed to the surface), in

particular selected from the group consisting of S12, S23, S45, S52, S53, S67, S103, S111, S119,

S126, S147, S214, S222, S320, S333, S336 and S393 (occupied by S residue), and more

preferably from the group consisting of S23, S52, S53, S67, T106, T108, S111, S119, S147,

15   S214 and T267 (having more than 50% of its side chain exposed to the surface), in particular

from the group consisting of S23, S52, S53, S67, S111, S119, S147 and S214 (occupied by an S

residue).

          While the non-polypeptide moiety of the conjugate according to this aspect of the

invention can be any molecule which, when using the given conjugation method has cysteine as

20   an attachment group it is preferred that the non-polypeptide moiety is a polymer molecule. The

polymer molecule can be any of the molecules mentioned in the section entitled "Conjugation to

a polymer molecule," but is preferably selected from the group consisting of linear or branched

polyethylene glycol or another polyalkylene oxide. In another embodiment, the polymer

molecule is PEG, such as VS-PEG. The conjugation between the polypeptide and the polymer

25   can be achieved in any suitable manner, e.g., as described in the section entitled "Conjugation to

a polymer molecule," e.g., in using a one step method or in the stepwise manner referred to in

said section. When the FVII or FVIIa polypeptide comprises only one conjugatable cysteine

residue, this is preferably conjugated to a non-polypeptide moiety with a molecular weight of

from about 5 kDa to about 20 kDa, e.g., from about 10 kDa to about 20 kDa, such as a molecular

30   weight of about 5 kDa, about 10 kDa, about 12 kDa, about 15 kDa or about 20 kDa, either

directly conjugated or indirectly through a low molecular weight polymer (as disclosed in WO

99/55377). When the conjugate comprises two or more conjugatable cysteine residue, normally

each of the non-polypeptide moieties has a molecular weight of from about 5 to about 10 kDa,

such as about 5 kDa or about 10 kDa.

5   It will be understood that any of the amino acid changes, in particular

substitutions, specified in this section can be combined with any of the amino acid changes,

preferably substitutions specified in the other sections herein disclosing specific amino acid

modifications, including introduction and/or removal of glycosylation sites.

Conjugate of the invention wherein the non-polypeptide moiety is a molecule that
10   **has aspartic acid or glutamic acid as an attachment group.**

           In a still further interesting embodiment of the invention, the non-polypeptide

moiety has aspartic acid or glutamic acid as an attachment group, i.e., the conjugate of the

invention is one which comprises at least one non-polypeptide moiety covalently attached to a

polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type

15   FVII or FVIIa shown in SEQ ID NO:1 in that at least one aspartic acid residue and/or at least one

glutamic acid residue has been introduced or removed.

           In a further interesting embodiment, the amino acid sequence of the FVII or FVIIa

polypeptide differs from that shown in SEQ ID NO:1 in that at least one aspartic acid residue

and/or glutamic acid residue, such as 1-15 aspartic acid residues and/or glutamic acid residues, in

20   particular 1-10, 1-6 or 2-4 aspartic acid residues and/or glutamic acid residues, has been

introduced, preferably by substitution.

           Examples of positions, wherein aspartic acid residues or glutamic acid residues

can be introduced include, but is not limited to, positions at or in the vicinity of the proteolytic

degradation sites described above.

25   Thus, in an interesting embodiment of the invention, the aspartic acid residue

and/or the glutamic acid residue to be introduced, preferably by substitution, is selected from the

group consisting of I30D/E, K32D/E, A34D/E, T37D/E, K38D/E, W41D/E, Y44D/E, S45D/E,

D46C, L141D/E, E142D/E, K143D/E, R144D/E, L288D/E, R290D/E, G291D/E, A292D/E,

Q313D/E, S314D/E, R315D/E, K316D/E, V317D/E, L390D/E, M391D/E, R392D/E, S393D/E,

30   P395D/E, R396D/E, P397D/E, G398D/E, V399D/E, L401D/E, R402D/E, A403D/E, P404D/E,

and combinations thereof, in particular selected from the group consisting of K32D/E, Y44D/E,

K143D/E, R290D/E, R315D/E, K341D/E, R392D/E, R396D/E, R402D/E and combinations

thereof.

           In addition to the above listed substitution(s), the polypeptide of the conjugate

35   according to the above embodiment can comprise removal, preferably by substitution, of at least

one of the aspartic acid residue and/or at least one glutamic acid residue.

26

5   Due to the relative high amount of lysine residues in the parent polypeptide it is

envisaged that at least one aspartic acid or glutamic acid residue should preferably be removed,

in particular by substitution, in order to avoid excessive conjugation to non-polypeptide moieties.

          Thus, in one embodiment, the amino acid sequence of the FVII or FVIIa

polypeptide part of the conjugate differs from that shown in SEQ ID NO:1 in that at least one

10   aspartic acid or glutamic acid residue, such as 1-15 aspartic acid or glutamic acid residues, in

particular 1-10, 1-6 or 2-4 aspartic acid or glutamic acid residues, has been removed, preferably

by substitution. For example, the aspartic acid and glutamic acid residue(s) to be removed,

preferably by substitution, is selected from the group consisting of D33, D46, D48, E77, E82,

D86, D87, E94, E99, D104, E116, D123, E132, E142, E163, D196, E210, D212, E215, D217,

15   D219, E220, D256, E265, E270, D289, E296, D309, D319, E325, D334, D338, D343, E385,

E394 and combinations thereof.

          It will be understood that it is particularly preferred that at least one of the aspartic

acid or glutamic acid residues, which are introduced at a predetermined site in the parent

molecule, is combined with at least one of the above-mentioned removals of aspartic acid or

20   glutamic acid residues. Thus, in a preferred embodiment of the invention, the amino acid

sequence of the FVII or FVIIa polypeptide part of the conjugate differs from that shown in SEQ

ID NO:1 in that at least one aspartic acid or glutamic acid residue has been removed, preferably

by substitution, and at least one aspartic acid or glutamic acid residue has been introduced,

preferably by substitution.

25   While the non-polypeptide moiety of the conjugate according to this aspect of the

invention, which has an aspartic acid group or a glutamic acid group as an attachment group, can

be any non-polypeptide moiety with such property, it is presently preferred that the non-

polypeptide moiety is a polymer molecule or an organic derivatizing agent, in particular a

polymer molecule, and the conjugate is prepared, e.g., as described by Sakane and Pardridge,

30   Pharmaceutical Research, Vol. 14, No. 8, 1997, pp 1085-1091.

          It will be understood that any of the amino acid changes, in particular

substitutions, specified in this section can be combined with any of the amino acid changes, in

particular substitutions specified in the other sections herein disclosing specific amino acid

changes, including introduction and/or removal of glycosylation sites.

<u>Conjugate of the invention wherein the non-polypeptide moiety is a sugar moiety</u>

In a further interesting embodiment of the invention, an attachment group for a sugar moiety, such as a glycosylation site, in particular an *in vivo* glycosylation site, has been inserted and/or removed.

Preferably, the conjugate of the invention is one which comprises at least one sugar moiety covalently attached to a polypeptide, wherein the amino acid sequence of the polypeptide differs from that of wild-type FVII or FVIIa shown in SEQ ID NO:1 in that at least one non-naturally occurring glycosylation site has been introduced and/or at least one naturally occurring glycosylation site has been removed. In particular, a non-naturally occurring glycosylation site has been introduced, or a non-naturally occurring glycosylation site has been introduced in combination with the removal of a natural occurring glycosylation site. The introduced glycosylation site can be an O-glycosylation site or an N-glycosylation site. Preferably the glycosylation site is an *in vivo* O-glycosylation site or an *in vivo* N-glycosylation site, in particular an *in vivo* N-glycosylation site.

When used in the present context, the term "naturally occurring glycosylation site" covers the glycosylation sites at postions N145, N322, S52 and S60. In a similar way, the term "naturally occurring *in vivo* O-glycosylation site" includes the positions S52 and S60, whereas the term "naturally occurring *in vivo* N-glycosylation site" includes positions N145 and N322.

Typically, the amino acid sequence of the FVII or FVIIa polypeptide differs from that shown in SEQ ID NO:1 in that at least one non-naturally occurring glycosylation site (e.g., at least one non-naturally occurring *in vivo* N-glycosylation site), such as 1-15 non-naturally occurring glycosylation sites (e.g., 1-15 non-naturally occurring *in vivo* N-glycosylation sites), in particular 1-10, 1-6 or 2-4 non-naturally occurring glycosylation sites (e.g., 1-10, 1-6 or 2-4 non-naturally occurring *in vivo* N-glycosylation sites), has been introduced, preferably by substitution.

It will be understood that in order to prepare a conjugate, wherein the polypeptide of the conjugate comprises one or more glycosylation sites, the polypeptide must be expressed in a host cell capable of attaching sugar (oligosaccharide) moieties at the glycosylation site(s) or alternatively subjected to *in vitro* glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to a sugar moiety."

5   In an interesting embodiment of this aspect, an *in vivo* glycosylation site is introduced into a position of the parent FVII or FVIIa molecule occupied by an amino acid residue exposed to the surface of the molecule, preferably with more than 25% of the side chain exposed to the solvent, in particular more than 50% exposed to the solvent (these positions are identified in the Methods section herein). The N-glycosylation site is then introduced in such a
10   way that the N-residue of said site is located in said position. Analogously, an O-glycosylation site is introduced so that the S or T residue making up such site is located in said position. Examples of such positions include K32S/T, I42S/T, Y44S/T, K143S/T, R290S/T, R315S/T, K341S/T, R392S/T, R396S/T, R402S/T and combinations thereof.

With respect to N-glycosylation, the *in vivo* glycosylation site is introduced into a
15   position wherein only one mutation is required to create the site (i.e., where any other amino acid residues required for creating a functional glycosylation site is already present in the molecule).

In other words, the conjugate according to the invention is preferably a conjugate, wherein the amino acid sequence of the polypeptide differs from SEQ ID NO:1 in that at least one naturally occurring N-X'-X sequence is substituted with a N-X'-S or N-X'-T sequence,
20   wherein X' is any amino acid except P, and X is any amino acid except for S and T.

In a similar way, the conjugate according to the invention is preferably a conjugate, wherein the amino acid sequence of the polypeptide differs from SEQ ID NO:1 in that at least one naturally occurring X-X'-S or X-X'-T sequence naturally present in SEQ ID NO:1 is substituted with a N-X'-S or a N-X'-T sequence, wherein X' is any amino acid except P, and X
25   is any amino acid except for N.

Specific examples of such substitutions creating an *in vivo* N-glycosylation site include a substitution selected from the group consisting of F4S/T, P10N, Q21N, W41N, S43N, A51N, G58N, L65N, G59S/T, E82S/T, N95S/T, G97S/T, Y101N, D104N, T106N, K109N, G117N, G124N, S126N, T128N, A175S/T, G179N, I186S/T, V188N, R202S/T, I205S/T,
30   D212N, E220N, I230N, P231N, P236N, G237N, V253N, E265N, T267N, E270N, R277N, L280N, G291N, P303S/T, L305N, Q312N, G318N, G331N, D334N, K337N, G342N, H348N, R353N, Y357N, I361N, V376N, R379N, M391N, and combinations thereof. Preferably, the substitution is selected from the group consisting of F4S/T, P10N, Q21N, W41N, A51N, G58N, G59S/T, N95S/T, G97S/T, Y101N, D104N, T106N, K109N, G117N, G124N, S126N, T128N,
35   A175S/T, I186S/T, V188N, R202S/T, I205S/T, D212N, E220N, V253N, E265N, T267N,

E270N, L280N, G291N, P303S/T, G318N, G331N, D334N, K337N, R353N, Y357N, M391N, and combinations thereof.

Alternatively, the *in vivo* glycosylation site is introduced in a position occupied by a lysine residue or an arginine residue, preferably occupied by an lysine residue, in particular so that the N-residue of the N-glycosylation site or the S or T residue of an O-glycosylation site substitutes the lysine residue.

Stated differently, the conjugate according to the invention is preferably a conjugate, wherein the amino acid sequence of the polypeptide differs from SEQ ID NO:1 in that at least one K-X'-X sequence or R-X'-X sequence, preferably a K-X'-X sequence, naturally present in SEQ ID NO:1 is substituted with a N-X'-S or a N-X'-T sequence, wherein X' is any amino acid except P, and X is any amino acid except for S and T.

Specific examples of such substitutions creating an *in vivo* N-glycosylation site include a substitution selected from the group consisting of K32N+A34S/T, K38N+F40S/T, K62N+Q64S/T, K85N+D87S/T, K137N+P139S/T, K143N+N145S/T, K148N+Q149S/T, K161N+E163S/T, K197N+K199S/T, K199N+W201S/T, K316N+G318S/T, K337N, K341N+D343S/T, K389N+M391S/T and combinations thereof.

In a further interesting embodiment of the glycosylation aspect described above, a glycosylation site, in particular an N-glycosylation site, can be introduced in positions at or in the vicinity of the proteolytic degradation sites described above (see the section entitled *"Conjugate of the invention"*).

Thus, specific examples of such substitutions creating an *in vivo* N-glycosylation site include a substitution selected from the group consisting of K32N+A34S/T, F31N+D33S/T, I30N+K32S/T, A34N+R36S/T, K38N+F40S/T, T37N+L39S/T, R36N+K38S/T, L39N+W41S/T, F40N+I42S/T, W41N, I42N+Y44S/T, S43N, Y44N+D46S/T, S45N+G47S/T, D46N+D48S/T, G47N+Q49S/T, K143N+N145S/T, E142N+R144S/T, L141N+K143S/T, I140N+E142S/T, R144N+A146S/T, A146N+K148S/T, S147N+P149S/T, R290N+A292S/T, D289N+G291S/T, L288N+R290S/T, L287N+D289S/T, G291N, A292N+A294S/T, T293N+L295S/T, R315N+V317S/T, S314N+K316S/T, Q313N+R315S/T, Q312N, K316N+G318S/T, V317N+D319S/T, G318N, K341N+D343S/T, S339N+K341S/T, G342N, D343N+G345S/T, R392N+E394S/T, M391N, L390N+R392S/T, K389N+M391S/T, S393N+P395S/T, E394N+R396S/T, P395N+P397S/T, R396N+G398S/T, P397N+V399S/T,

5   G398N+L400S/T, V399N+L401S/T, L400N+R402S/T, L401N+A403S/T, R402N+P404S/T,
     A403N+F405S/T, P404N+P406S/T and combinations thereof, such as K143N+N145S/T+
     R315N+V317S/T. Preferably, the substitution is selected from the group consisting of
     K32N+A34S/T, K38N+F40S/T, Y44N+D46S/T, K143N+N145S/T, R290N+A292S/T,
     R315N+V317S/T, K341N+D343S/T, R392N+E394S/T, R396N+G398S/T, R402N+P404S/T

10   and combinations thereof, such as K143N+N145S/T+ R315N+V317S/T. More preferably, the
     substitution is selected from the group consisting of K32N+A34T, K38N+F40T, Y44N+D46T,
     K143N+N145T, R290N+A292T, R315N+V317T, 341N+D343T, R392N+E394T,
     R396N+G398T, R402N+P404T and combinations thereof, in particular K143N+N145T+
     R315N+V317T.

15   In a still further interesting embodiment of the invention, it is preferred that the *in
     vivo* glycosylation site is introduced in a position which does neither form part of the tissue
     factor binding nor form part of the active site region and the ridge of the active site binding cleft
     as defined herein. It is envisaged that such glycosylation variants will primarily belong to the
     class of active conjugates as defined hereinbefore.

20   Thus, specific examples of substitutions creating such an *in vivo* N-glycosylation
     site include substitutions selcted from the group consisting of K32N+A34S/T, I30N+K32S/T,
     A34N+R36S/T, K38N+F40S/T, T37N+L39S/T, W41N, Y44N+D46S/T, S45N+G47S/T,
     D46N+D48S/T, G47N+Q49S/T, K143N+N145S/T, E142N+R144S/T, L141N+K143S/T,
     I140N+E142S/T, R144N+A146S/T, A146N+K148S/T, S147N+P149S/T, L288N+R290S/T,

25   L287N+D289S/T, R315N+V317S/T, S314N+K316S/T, K316N+G318S/T, V317N+D319S/T,
     G318N, R392N+E394S/T, M391N, L390N+R392S/T, K389N+M391S/T, S393N+P395S/T,
     E394N+R396S/T, P395N+P397S/T, R396N+G398S/T, P397N+V399S/T, G398N+L400S/T,
     V399N+L401S/T, L401N+A403S/T, R402N+P404S/T, A403N+F405S/T, P404N+P406S/T and
     combinations thereof, such as K143N+N145S/T+ R315N+V317S/T. Preferably, the substitution

30   is selcted from the group consisting of K32N+A34S/T, K38N+F40S/T, Y44N+D46S/T,
     K143N+N145S/T, R315N+V317S/T, R392N+E394S/T, R396N+G398S/T, R402N+P404S/T
     and combinations thereof, such as K143N+N145S/T+ R315N+V317S/T. More preferably, the
     substitution is selected from the group consisting of K32N+A34T, K38N+F40T, Y44N+D46T,
     K143N+N145T, R315N+V317T, R392N+E394T, R396N+G398T, R402N+P404T and

35   combinations thereof, in particular K143N+N145T+ R315N+V317T.

5   In an even further interesting embodiment of the invention, it is preferred that the

in vivo glycosylation site is introduced in a position which does not form part of the tissue factor

but which forms part of the active site region and the ridge of the active site binding cleft as

defined herein. It is envisaged that such glycosylation variants will primarily belong to the class

of inactive conjugates as defined hereinbefore.

10   Thus, specific examples of substitutions creating such an *in vivo* N-glycosylation

site include substitutions selcted from the group consisting of I153N+G155S/T,

Q167N+L169S/T, V168N+L170S/T, L169N+L171S/T, L170N+V172S/T, L171N+N173S/T,

A175S/T, A175N+L177S/T, L177N+G179S/T, G179N, G180N+L182S/T, T181N+I183S/T,

V188N, V189N+A191S/T, S190N+A192S/T, A191N+H193S/T, H193N+F195S/T,

15   F195N+K197S/T, D196N+I198S/T, K197N+K199S/T, I198N+N200S/T, K199N+W201S/T,

W201N+N203S/T, R202S/T, I205S/T, V228N+I230S/T, I229N+P231S/T, I230N, P231N,

S232N+Y234S/T, T233N+V235S/T, Y234N+P236S/T, V235N+G237S/T, P236N, G237N,

T238N+N240S/T, T239N+H241S/T, H241N+I243S/T, D242S/T, I243N+L245S/T,

A244N+L246S/T, L245N+R247S/T, L246N+L246S/T, V281N+G283S/T, S282N+W284S/T,

20   G283N+G285S/T, W284N+Q286S/T, G285N+L287S/T, Q286N+L288S/T, D289N+G291S/T,

R290N+A292S/T, G291N, A292N+A294S/T, T293N+L295S/T, P321N+I323S/T,

T324N+Y326S/T, E325N+M327S/T, Y326N+F327S/T, F328N+A330S/T, S339N+K341S/T,

K341N+D343S/T, G342N+S344S/T, D343N+G345S/T, S344N+G346S/T, G345N+P347S/T,

P347N+A349S/T, H348N, L358N+G360S/T, T359N+I361S/T, G360N+V362S/T, I361N,

25   V362N+W364S/T, S363N+G365S/T, W364N+Q366S/T, G365N+G367S/T, T370N+G372S/T,

V376N, Y377N+R379S/T, T378N+V380S/T, R379N, V380N+Q382S/T, Q382N+I384S/T,

Y383N+E385S/T, W386N+Q388S/T, L387N+K389S/T, L400N+R402S/T and combinations

thereof. Preferably, the substitution is selected from the group consisting of D289N+G291S/T,

R290N+A292S/T, G291N, A292N+A294S/T, T293N+L295S/T, S339N+K341S/T,

30   K341N+D343S/T, G342N+S344S/T, D343N+G345S/T, and combinations thereof. More

preferably, the substitution is selcted from the group consisting of D289N+G291T,

R290N+A292T, G291N, A292N+A294T, T293N+L295T, S339N+K341T, K341N+D343T,

G342N+S344T, D343N+G345T, and combinations thereof.

          In addition to a sugar moiety, the conjugate according to the aspect of the

35   invention described in the present section can contain additional non-polypeptide moieties, in

5   particular a polymer molecule, as described in the present application, conjugated to one or more
       attachment groups present in the polypeptide part of the conjugate.

              It will be understood that any of the amino acid changes, in particular
       substitutions, specified in this section can be combined with any of the amino acid changes, in
       particular substitutions, specified in the other sections herein disclosing specific amino acid
10   changes.

              For instance, any of the glycosylated variants disclosed in the present section
       having introduced and/or removed at least one glycosylation site, such as a variant comprising
       the substitutions R315N + V317T and/or K143N + N145T, can further be conjugated to a
       polymer molecule, such as PEG, or any other non-polypeptide moiety. For this purpose the
15   conjugation can be achieved by use of attachment groups already present in the FVII or FVIIa
       polypeptide or attachment groups can have been introduced and/or removed, in particular such
       that a total of 1-6, in particular 3-4 or 1, 2, 3, 4, 5, or 6 attachment groups are available for
       conjugation.

              Preferably, in a conjugate of the invention wherein the FVII or FVIIa polypeptide
20   comprises two glycosylation sites, the number and molecular weight of the non-polypeptide
       moiety is chosen so as that the total molecular weight added by the non-polypeptide moiety is in
       the range of 5-25 kDa, such as in the range of 10-25 kDa, in particular about 5 kDa, about 12
       kDa, about 15 kDa or about 20kDa.

              An inactive conjugate
25   The conjugates of the invention can be rendered inactive by removing at least one
       amino acid residue occupying a position selected from the group consisting of R152, I153, S344,
       D242 and H193 of SEQ ID NO:1. The removal can be effected by substitution or deletion of one
       or more of the above-identified amino acid residues. Preferably, the removal is effected by
       substitution, in particular by conservative substitution. Accordingly, the inactive FVII or FVIIa
30   polypeptide used herein can comprise one or more of the following substitutions: R152X, I153X,
       S344X, D242X or H193X, wherein X is any amino acid residue, preferably one leading to a
       conservative substitution. For instance, the inactive FVII or FVIIa polypeptide comprises the
       mutations R152X, wherein X is any amino acid residue other than lysine (since lysine forms part
       of a protease cleavage site). Other examples of specific substitutions include I153A/V/L;
35   S344T/A/G/Y; D242E/A and/or H193R/A.

5   Alternatively, an active FVII or FVIIa polypeptide can be rendered inactive by

carbamylating the α-amino acid group I153 or by complexing the polypeptide to a serine

proteinase inhibitor. A suitable serine inhibitor protein is, e.g., selected from the group consisting

of an organophosphor coumpound, a sulfanylfluoride, a peptide halomethylketone, preferably a

Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-Glu-Glu-Arg chlormethylketone, Dansyl-Phe-

10   Phe-Arg chlormethylketone or a Phe-Phe-Arg chlormethylketone, or an azapeptide.

A conjugate can also be rendered inactive by introducing at least one

glycosylation site in a position selected so that the subsequent glycosylation inactivates the

conjugate.

As explained above in the last part of the section entitled *"Conjugate of the*

15   *invention wherein the non-polypeptide moiety is a sugar moiety"* it is preferred that such

glycosylation sites are introduced in a position which does not form part of the tissue factor but

which forms part of the active site region and the ridge of the active site binding cleft as defined

herein. Specific examples of preferred substitutions ar egiven above in the section entitled

"Conjugate of the invention wherein the non-polypeptide moiety is a sugar moiety".

Non-polypeptide moiety of the conjugate of the invention

20   As indicated further above the non-polypeptide moiety of the conjugate of the

invention is preferably selected from the group consisting of a polymer molecule, a lipophilic

compound, a sugar moiety (by way of *in vivo* glycosylation) and an organic derivatizing agent.

All of these agents can confer desirable properties to the polypeptide part of the conjugate, in

25   particular increased functional *in vivo* half-life and/or increased plasma half-life. The polypeptide

part of the conjugate is normally conjugated to only one type of non-polypeptide moiety, but can

also be conjugated to two or more different types of non-polypeptide moieties, e.g., to a polymer

molecule and a sugar moiety, to a lipophilic group and a sugar moiety, to an organic derivatizing

agent and a sugar moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to

30   two or more different non-polypeptide moieties can be done simultaneous or sequentially.

Methods of preparing a conjugate of the invention

In the following sections "Conjugation to a lipophilic compound," "Conjugation

to a polymer molecule," "Conjugation to a sugar moiety" and "Conjugation to an organic

derivatizing agent" conjugation to specific types of non-polypeptide moieties is described. In

35   general, a polypeptide conjugate according to the invention can be produced by culturing an

5   appropriate host cell under conditions conducive for the expression of the polypeptide, and recovering the polypeptide, wherein a) the polypeptide comprises at least one N- or O-glycosylation site and the host cell is a eukaryotic host cell capable of *in vivo* glycosylation, and/or b) the polypeptide is subjected to conjugation to a non-polypeptide moiety *in vitro*.

It will be understood that the conjugation should be designed so as to produce the

10   optimal molecule with respect to the number of non-polypeptide moieties attached, the size and form of such molecules (e.g., whether they are linear or branched), and the attachment site(s) in the polypeptide. The molecular weight of the non-polypeptide moiety to be used can e.g., be chosen on the basis of the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight (e.g., to reduce renal

15   clearance) it is usually desirable to conjugate as few high molecular weight non-polypeptide moieties as possible to obtain the desired molecular weight. When a high degree of shielding is desirable this can be obtained by use of a sufficiently high number of low molecular weight non-polypeptide moieties (e.g., with a molecular weight of from about 300 Da to about 5 kDa, such as a molecular weight of from 300 Da to 2 kDa) to effectively shield all or most protease

20   cleavage sites or other vulnerable sites of the polypeptide.

Conjugation to a lipophilic compound
The polypeptide and the lipophilic compound can be conjugated to each other, either directly or by use of a linker. The lipophilic compound can be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine,

25   a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the polypeptide and the lipophilic compound, optionally through a linker can be done according to methods known in the art, e.g., as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

30   Conjugation to a polymer molecule, including conjugation of a polymer molecule to the N-terminal of the polypeptide
The polymer molecule to be coupled to the polypeptide can be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of about 300-100,000 Da, such as about 500-20,000 Da,

35   more preferably in the range of about 500-15,000 Da, even more preferably in the range of about 2-12 kDa, such as in the range of about 3-10 kDa. When the term "about" is used herein in

5   connection with a certain molecular weight, the word "about" indicates an approximate average
molecular weight and reflects the fact that there will normally be a certain molecular weight
distribution in a given polymer preparation.

Examples of homo-polymers include a polyol (i.e., poly-OH), a polyamine (i.e.,
poly-NH$_2$) and a polycarboxylic acid (i.e., poly-COOH). A hetero-polymer is a polymer
10   comprising different coupling groups, such as a hydroxyl group and an amine group.

Examples of suitable polymer molecules include polymer molecules selected
from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG),
such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl
alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid
15   anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or
any other biopolymer suitable for reducing immunogenicity and/or increasing functional *in vivo*
half-life and/or serum half-life. Another example of a polymer molecule is human albumin or
another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are
biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility
20   properties, and are easily excreted from living organisms.

PEG is the preferred polymer molecule, since it has only few reactive groups
capable of cross-linking compared to, e.g., polysaccharides such as dextran. In particular, mono-
functional PEG, e.g., methoxypolyethylene glycol (mPEG), is of interest since its coupling
chemistry is relatively simple (only one reactive group is available for conjugating with
25   attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the
resulting polypeptide conjugates are more homogeneous and the reaction of the polymer
molecules with the polypeptide is easier to control.

To effect covalent attachment of the polymer molecule(s) to the polypeptide, the
hydroxyl end groups of the polymer molecule must be provided in activated form, i.e., with
30   reactive functional groups (examples of which include primary amino groups, hydrazide (HZ),
thiol, succinate (SUC), succinimidyl succinate (SS), succinimidyl succinamide (SSA),
succinimidyl proprionate (SPA), succinimidy carboxymethylate (SCM), benzotriazole carbonate
(BTC), N-hydroxysuccinimide (NHS), aldehyde, nitrophenylcarbonate (NPC), and tresylate
(TRES)). Suitable activated polymer molecules are commercially available, e.g., from
35   Shearwater Polymers, Inc., Huntsville, AL, USA, or from PolyMASC Pharmaceuticals plc, UK.

5   Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g., as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference).

10   Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g., SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG, BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in US 5,932,462 and US 5,643,575, both of which are incorporated

15   herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: US 5,824,778, US 5,476,653, WO 97/32607, EP 229,108, EP 402,378, US 4,902,502, US 5,281,698, US 5,122,614, US 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO

20   97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, US 5,736,625, WO 98/05363, EP 809 996, US 5,629,384, WO 96/41813, WO 96/07670, US 5,473,034, US 5,516,673, EP 605 963, US 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

25   The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g., as described in the following references (which also describe suitable methods for activation of polymer molecules): R.F. Taylor, (1991), "Protein immobilisation. Fundamental and applications," Marcel Dekker, N.Y.; S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking," CRC Press, Florida, USA; G.T.

30   Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques," Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g., being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation can

35   be directed towards conjugation to all available attachment groups on the polypeptide (i.e., such

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5   attachment groups that are exposed at the surface of the polypeptide) or can be directed towards
one or more specific attachment groups, e.g., the N-terminal amino group as described in US
5,985,265. Furthermore, the conjugation can be achieved in one step or in a stepwise manner
(e.g., as described in WO 99/55377).

It will be understood that the PEGylation is designed so as to produce the optimal
10   molecule with respect to the number of PEG molecules attached, the size and form of such
molecules (e.g., whether they are linear or branched), and the attachment site(s) in the
polypeptide. The molecular weight of the polymer to be used can e.g., be chosen on the basis of
the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to
achieve a conjugate having a high molecular weight (e.g., to reduce renal clearance) it is usually
15   desirable to conjugate as few high molecular weight polymer molecules as possible to obtain the
desired molecular weight. When a high degree of shielding is desirable this can be obtained by
use of a sufficiently high number of low molecular weight polymer molecules (e.g., with a
molecular weight of from about 300 Da to about 5 kDa) to effectively shield all or most protease
cleavage sites or other vulnerable sites of the polypeptide. For instance, 2-8, such as 3-6 such
20   polymers can be used.

In connection with conjugation to only a single attachment group on the protein
(e.g., the N-terminal amino group), it can be advantageous that the polymer molecule, which can
be linear or branched, has a high molecular weight, preferably about 10-25 kDa, such as about
15-25 kDa, e.g., about 20 kDa.
25   Normally, the polymer conjugation is performed under conditions aimed at
reacting as many of the available polymer attachment groups with polymer molecules. This is
achieved by means of a suitable molar excess of the polymer relative to the polypeptide.
Typically, the molar ratios of activated polymer molecules to polypeptide are up to about 1000-1,
such as up to about 200-1, or up to about 100-1. In some cases the ration can be somewhat lower,
30   however, such as up to about 50-1, 10-1 or 5-1 in order to obtain optimal reaction.

It is also contemplated according to the invention to couple the polymer
molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled
person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem.,
252, 3578-3581; US 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. Ed., 24,
35   375-378).

5   Subsequent to the conjugation, residual activated polymer molecules are blocked according to methods known in the art, e.g., by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules are removed by a suitable method.

It will be understood that depending on the circumstances, e.g., the amino acid sequence of the polypeptide, the nature of the activated PEG compound being used and the specific PEGylation conditions, including the molar ratio of PEG to polypeptide, varying degrees

10   of PEGylation can be obtained, with a higher degree of PEGylation generally being obtained with a higher ratio of PEG to polypeptide. The PEGylated polypeptides resulting from any given PEGylation process will, however, normally comprise a stochastic distribution of polypeptide conjugates having slightly different degrees of PEGylation.

15   In an interesting embodiment of the invention, the polypeptide conjugate of the invention comprises a polymer molecule covalently attached to the A1 N-terminal of the wild type FVII or FVIIa polypeptide shown in SEQ ID NO:1, where said polymer molecule is the only polymer molecule attached to the polypeptide. Preferably, such polypeptide conjugates are ones, which comprise a single PEG molecule attached to the N-terminal of the polypeptide and

20   no other PEG molecules. In particular, a linear or branched PEG molecule with a molecular weight of at least about 5 kDa, in particular about 10-25 kDa, such as about 15-25 kDa, e.g., about 20 kDa is preferred. The polypeptide conjugate according to this embodiment can further comprise one or more sugar moieties attached to an N-linked or O-linked glycosylation site of the polypeptide or sugar moieties attached by *in vitro* glycosylation.

25   In a further interesting embodiment of the invention the polypeptide conjugate of the invention comprises polymer molecules covalently attached to the A1 N-terminal and to the I153 N-terminal of the wild type FVIIa polypeptide shown in SEQ ID NO:1, where said polymer molecules are the only polymer molecules attached to the polypeptide. Preferably, such polypeptide conjugates are ones, which comprise a PEG molecule attached to both of the N-

30   terminals of FVIIa and no other PEG molecules. In particular, linear or branched PEG molecules with a molecular weight of at least about 5 kDa, in particular about 10-25 kDa, such as about 15-25 kDa, e.g., about 20 kDa are preferred. The polypeptide conjugate according to this embodiment can further comprise one or more sugar moieties attached to an N-linked or O-linked glycosylation site of the polypeptide or sugar moieties attached by *in vitro* glycosylation.

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5   One preferred method for selectively coupling polymer molecules, such as PEG

molecules, to the N-terminal of the polypeptide is the method disclosed in US 5,985,265. This

method involves reductive alkylation (reaction of the N-terminal amino group of the polypeptide

with an aldehyde-containing polypeptide, such as aldehylde-PEG, in the presence of a reducing

agent, such as NaCNBH$_3$). This method exploits differential reactivity of different types of

10   primary amino groups (lysine versus N-terminal) available for derivatization in the polypeptide,

thereby achieving substantially selective derivatization of the polypeptide at the N-terminus with

a carbonyl group-containing polymer molecule, such as aldehyde-PEG. The reaction is

performed at a pH which allows one to take advantage of the pK$_a$ differences between the ε-

amino groups of the lysine residues and that of the α-amino group of the N-terminal residue of

15   the polypeptide. In order to achieve this differential reactivity, the reaction is typically carried at

slightly acidic conditions. Specific examples of suitable pH ranges include pH 4.5-7, such as pH

4.5-6, e.g., pH 5-6, in particular about pH 5.

In another specific embodiment, the polypeptide conjugate of the invention

comprises a PEG molecule attached to each of the lysine residues in the polypeptide available for

20   PEGylation, in particular a linear or branched PEG molecule, e.g., with a molecular weight of

about 1-15 kDa, typically about 2-12 kDa, such as about 3-10 kDa, e.g., about 5 or 6 kDa.

In yet another embodiment, the polypeptide conjugate of the invention comprises

a PEG molecule attached to each of the lysine residues in the polypeptide available for

PEGylation, and in addition to the N-terminal amino acid residue of the polypeptide.

25   Covalent *in vitro* coupling of carbohydrate moieties (such as dextran) to amino

acid residues of the polypeptide can also be used, e.g., as described, for example in WO

87/05330 and in Aplin etl al., CRC Crit Rev. Biochem, pp. 259-306, 1981. The *in vitro* coupling

of carbohydrate moieties or PEG to protein- and peptide-bound Gln-residues can be carried out

by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups

30   to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-

amine groups can be protein- or peptide-bound , such as the ε-amino-group in Lys-residues or it

can be part of a small or large organic molecule. An example of a small organic molecule

functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane).

An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-

35   linking is an amine-containing PEG (Sato et al., 1996, Biochemistry 35, 13072-13080).

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5   TGases, in general, are highly specific enzymes, and not every Gln-residues
exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-
containing substances. On the contrary, only few Gln-residues are naturally functioning as TGase
substrates but the exact parameters governing which Gln-residues are good TGase substrates
remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking
10   reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence
known to function very well as TGase substrates. Several amino acid sequences are known to be
or to contain excellent natural TGase substrates e.g., substance P, elafin, fibrinogen, fibronectin,
α_2-plasmin inhibitor, α-caseins, and β-caseins.

Coupling to a sugar moiety
15   In order to achieve *in vivo* glycosylation of a FVII molecule comprising one or
more glycosylation sites the nucleotide sequence encoding the polypeptide must be inserted in a
glycosylating, eucaryotic expression host. The expression host cell can be selected from fungal
(filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one
embodiment, the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g., HEK
20   293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g., *S. cerevisiae* or *Pichia
pastoris*, or any of the host cells mentioned hereinafter.

Coupling to an organic derivatizing agent
       Covalent modification of the polypeptide can be performed by reacting one or
more attachment groups of the polypeptide with an organic derivatizing agent. Suitable
25   derivatizing agents and methods are well known in the art. For example, cysteinyl residues most
commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid
or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl
residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-
imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl
30   disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol,
or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with
diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side
chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M
sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or
35   other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the

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5   charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing

residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal,

chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione and

transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with

one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-

10   cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction

be performed in alkaline conditions because of the high pKa of the guanidine functional group.

Furthermore, these reagents can react with the groups of lysine as well as the

arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified

by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such

15   as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-

dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to

asparaginyl and glutaminyl residues by reaction with ammonium ions.

Blocking of functional site

It has been reported that excessive polymer conjugation can lead to a loss of

20   activity of the polypeptide to which the non-polypeptide moiety is conjugated. This problem can

be eliminated, e.g., by removal of attachment groups located at the functional site or by

reversible blocking the functional site prior to conjugation so that the functional site is blocked

during conjugation. The latter strategy constitutes further embodiments of the invention (the first

strategy being exemplified further above, e.g., by removal of lysine residues which can be

25   located close to the functional site). More specifically, according to the second strategy the

conjugation between the polypeptide and the non-polypeptide moiety is conducted under

conditions where the functional site of the polypeptide is blocked by a helper molecule e.g.,

tissue factor capable of binding to the functional site of the polypeptide or a serine protease

inhibitor.

30   Preferably, the helper molecule is one, which specifically recognizes a functional

site of the polypeptide, such as a receptor, in particular tissue factor, either full length or a

suitably truncated form of tissue factor or two molecules, one being tissue factor the other one

being a peptide or peptide inhibitor binding to and thus protecting the area around the catalytic

triad (preferably defined as amino acid residues within 10 Å of any atom in the catalytic triad).

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5   Alternatively, the helper molecule can be an antibody, in particular a monoclonal

antibody recognizing the FVII polypeptide. In particular, the helper molecule can be a

neutralizing monoclonal antibody.

           The polypeptide is allowed to interact with the helper molecule before effecting

conjugation. This ensures that the functional site of the polypeptide is shielded or protected and

10   consequently unavailable for derivatization by the non-polypeptide moiety such, as a polymer.

Following its elution from the helper molecule, the conjugate between the non-polypeptide

moiety and the polypeptide can be recovered with at least a partially preserved functional site.

           The subsequent conjugation of the polypeptide having a blocked functional site to

a polymer, a lipophilic compound, a sugar moiety, an organic derivatizing agent or any other

15   compound is conducted in the normal way, e.g., as described in the sections above entitled

"Conjugation to ....".

           Irrespectively of the nature of the helper molecule to be used to shield the

functional site of the polypeptide from conjugation, it is desirable that the helper molecule is free

from or comprises only few attachment groups for the non-polypeptide moiety of choice in

20   part(s) of the molecule, where the conjugation to such groups will hamper the desorption of the

conjugated polypeptide from the helper molecule. Hereby, selective conjugation to attachment

groups present in non-shielded parts of the polypeptide can be obtained and it is possible to reuse

the helper molecule for repeated cycles of conjugation. For instance, if the non-polypeptide

moiety is a polymer molecule such as PEG, which has the epsilon amino group of a lysine or N-

25   terminal amino acid residue as an attachment group, it is desirable that the helper molecule is

substantially free from conjugatable epsilon amino groups, preferably free from any epsilon

amino groups. Accordingly, in a preferred embodiment, the helper molecule is a protein or

peptide capable of binding to the functional site of the polypeptide, which protein or peptide is

free from any conjugatable attachment groups for the non-polypeptide moiety of choice.

30   In a further embodiment, the helper molecule is first covalently linked to a solid

phase such as column packing materials, for instance Sephadex or agarose beads, or a surface,

e.g., reaction vessel. Subsequently, the polypeptide is loaded onto the column material carrying

the helper molecule and conjugation carried out according to methods known in the art, e.g., as

described in the sections above entitled "Conjugation to ....." This procedure allows the

35   polypeptide conjugate to be separated from the helper molecule by elution. The polypeptide

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5   conjugate is eluted by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the polypeptide conjugate. The fluid phase containing the polypeptide conjugate is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other ways: For instance, the helper molecule can be derivatised with a second molecule (e.g., biotin) that can be recognized by a

10   specific binder (e.g., streptavidin). The specific binder can be linked to a solid phase thereby allowing the separation of the polypeptide conjugate from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the helper molecule-second molecule complex, but not the polypeptide conjugate. The polypeptide conjugate can be released from the helper molecule in any

15   appropriate fashion. Deprotection can be achieved by providing conditions in which the helper molecule dissociates from the functional site of the FVII to which it is bound. For instance, a complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH to an acid or alkaline pH. Even more preferred is the use of a conformation specific antibody that recognizes a Ca^{2+} specific conformation of FVII and

20   consequently can be eluted with EDTA under mild conditions.

ATTACHMENT OF SERINE PROTEASE INHIBITOR

Attachment of a serine protease inhibitor can be performed in accordance with the method described in WO 96/12800.

Conjugation of a tagged polypeptide

25   In an alternative embodiment, the polypeptide is expressed as a fusion protein with a tag, i.e., an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide and the non-polypeptide moiety. In particular, the tag can be used for achieving conjugation in microtiter plates or other carriers,

30   such as paramagnetic beads, to which the tagged polypeptide can be immobilised via the tag. The conjugation to the tagged polypeptide in, e.g., microtiter plates has the advantage that the tagged polypeptide can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag can function

35   as a spacer molecule, ensuring an improved accessibility to the immobilised polypeptide to be

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5   conjugated. The conjugation using a tagged polypeptide can be to any of the non-polypeptide

moieties disclosed herein, e.g., to a polymer molecule such as PEG.

The identity of the specific tag to be used is not critical as long as the tag is

capable of being expressed with the polypeptide and is capable of being immobilised on a

suitable surface or carrier material. A number of suitable tags are commercially available, e.g.,

10   from Unizyme Laboratories, Denmark. For instance, the tag can consist of any of the following

sequences:

His-His-His-His-His-His

Met-Lys-His-His-His-His-His-His

Met-Lys-His-His-Ala-His-His-Gln-His-His

15   Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln

Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-Gln

or any of the following:

EQKLI SEEDL (a C-terminal tag described in Mol. Cell. Biol. 5:3610-16, 1985)

DYKDDDDK (a C- or N-terminal tag)

20   YPYDVPDYA

Antibodies against the above tags are commercially available, e.g., from ADI,

Aves Lab and Research Diagnostics.

The subsequent cleavage of the tag from the polypeptide can be achieved by use

of commercially available enzymes.

25   Methods of preparing a polypeptide of the invention or the polypeptide of the
conjugate of the invention

The polypeptide of the present invention or the polypeptide part of a conjugate of

the invention, optionally in glycosylated form, can be produced by any suitable method known in

the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and

30   expressing the sequence in a suitable transformed or transfected host. Preferably, the host cell is

a gammacarboxylating host cell such as a mammalian cell. However, polypeptides of the

invention can be produced, albeit less efficiently, by chemical synthesis or a combination of

chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

A nucleotide sequence encoding a polypeptide or the polypeptide part of a

35   conjugate of the invention can be constructed by isolating or synthesizing a nucleotide sequence

encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO:1

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5 and then changing the nucleotide sequence so as to effect introduction (i.e., insertion or substitution) or removal (i.e., deletion or substitution) of the relevant amino acid residue(s).

The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g., by using an oligonucleotide synthesizer, wherein oligonucleotides are

10 designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3'

15 overhangs for complementary assembly.

Alternative nucleotide sequence modification methods are available for producing polypeptide variants for high throughput screening, for instance methods which involve homologous cross-over such as disclosed in US 5,093,257, and methods which involve gene shuffling, i.e., recombination between two or more homologous nucleotide sequences resulting

20 in new nucleotide sequences having a number of nucleotide alterations when compared to the starting nucleotide sequences. Gene shuffling (also known as DNA shuffling) involves one or more cycles of random fragmentation and reassembly of the nucleotide sequences, followed by screening to select nucleotide sequences encoding polypeptides with desired properties. In order for homology-based nucleic acid shuffling to take place, the relevant parts of the nucleotide

25 sequences are preferably at least 50% identical, such as at least 60% identical, more preferably at least 70% identical, such as at least 80% identical. The recombination can be performed *in vitro* or *in vivo*.

Examples of suitable *in vitro* gene shuffling methods are disclosed by Stemmer et al. (1994), Proc. Natl. Acad. Sci. USA; vol. 91, pp. 10747-10751; Stemmer (1994), Nature, vol.

30 370, pp. 389-391; Smith (1994), Nature vol. 370, pp. 324-325; Zhao et al., Nat. Biotechnol. 1998, Mar; 16(3): 258-61; Zhao H. and Arnold, FB, Nucleic Acids Research, 1997, Vol. 25. No. 6 pp. 1307-1308; Shao et al., Nucleic Acids Research 1998, Jan 15; 26(2): pp. 681-83; and WO 95/17413.

An example of a suitable *in vivo* shuffling method is disclosed in WO 97/07205.

35 Other techniques for mutagenesis of nucleic acid sequences by *in vitro* or *in vivo* recombination

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5   are disclosed e.g., in WO 97/20078 and US 5,837,458. Examples of specific shuffling techniques
     include "family shuffling," "synthetic shuffling" and "*in silico* shuffling".

     Family shuffling involves subjecting a family of homologous genes from different
     species to one or more cycles of shuffling and subsequent screening or selection. Family
     shuffling techniques are disclosed e.g., by Crameri et al. (1998), Nature, vol. 391, pp. 288-291;
10   Christians et al. (1999), Nature Biotechnology, vol. 17, pp. 259-264; Chang et al. (1999), Nature
     Biotechnology, vol. 17, pp. 793-797; and Ness et al. (1999), Nature Biotechnology, vol. 17, 893-
     896.

     Synthetic shuffling involves providing libraries of overlapping synthetic
     oligonucleotides based e.g., on a sequence alignment of homologous genes of interest. The
15   synthetically generated oligonucleotides are recombined, and the resulting recombinant nucleic
     acid sequences are screened and if desired used for further shuffling cycles. Synthetic shuffling
     techniques are disclosed in WO 00/42561.

     In silico shuffling refers to a DNA shuffling procedure, which is performed or
     modelled using a computer system, thereby partly or entirely avoiding the need for physically
20   manipulating nucleic acids. Techniques for *in silico* shuffling are disclosed in WO 00/42560.

     Once assembled (by synthesis, site-directed mutagenesis or another method), the
     nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably
     linked to control sequences necessary for expression of the FVII in the desired transformed host
     cell.

25   It should of course be understood that not all vectors and expression control
     sequences function equally well to express the nucleotide sequence encoding a polypeptide
     described herein. Neither will all hosts function equally well with the same expression system.
     However, one of skill in the art can make a selection among these vectors, expression control
     sequences and hosts without undue experimentation. For example, in selecting a vector, the host
30   must be considered because the vector must replicate in it or be able to integrate into the
     chromosome. The vector's copy number, the ability to control that copy number, and the
     expression of any other proteins encoded by the vector, such as antibiotic markers, should also
     be considered. In selecting an expression control sequence, a variety of factors should also be
     considered. These include, for example, the relative strength of the sequence, its controllability,
35   and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as

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5   regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

10   The recombinant vector can be an autonomously replicating vector, i.e., a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

15   The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jola, CA, USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad.

25   Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells," Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from E. coli, including

30   pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such amplifiable vectors are

35   well known in the art. They include, for example, vectors able to be amplified by DHFR

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5   amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction
       of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient
       Expression," Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS")
       amplification (see, e.g., US 5,122,464 and EP 338,841).

               The recombinant vector can further comprise a DNA sequence enabling the
10   vector to replicate in the host cell in question. An example of such a sequence (when the host cell
       is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable
       sequences enabling the vector to replicate are the yeast plasmid 2µ replication genes REP 1-3
       and origin of replication.

               The vector can also comprise a selectable marker, e.g., a gene the product of
15   which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase
       (DHFR) or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40,
       1985, pp. 125-130), or one which confers resistance to a drug, e.g., ampicillin, kanamycin,
       tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces*
       cerevisiae, selectable markers include *ura3 and leu2*. For filamentous fungi, selectable markers
20   include *amdS*, *pyrG*, *arcB*, *niaD* and *sC*.

               The term "control sequences" is defined herein to include all components, which
       are necessary or advantageous for the expression of the polypeptide of the invention. Each
       control sequence can be native or foreign to the nucleic acid sequence encoding the polypeptide.
       Such control sequences include, but are not limited to, a leader sequence, polyadenylation
25   sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal
       peptide sequence, and transcription terminator. At a minimum, the control sequences include a
       promoter.

               A wide variety of expression control sequences can be used in the present
       invention. Such useful expression control sequences include the expression control sequences
30   associated with structural genes of the foregoing expression vectors as well as any sequence
       known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and
       various combinations thereof.

               Examples of suitable control sequences for directing transcription in mammalian
       cells include the early and late promoters of SV40 and adenovirus, e.g., the adenovirus 2 major
35   late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus

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5   immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the

Drosophila minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter,

the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or

adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J*

Mol Biol 1987 Aug 20;196(4):947-50).

10   In order to improve expression in mammalian cells a synthetic intron can be

inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An

example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from

Promega Corporation, WI, USA).

                Examples of suitable control sequences for directing transcription in insect cells

15   include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis

virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the

baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence.

Examples of suitable control sequences for use in yeast host cells include the promoters of the

yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from

20   yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the

inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal

host cells include the ADH3 promoter and terminator, a promoter derived from the genes

encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an

A. niger α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor*

25   *miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of

suitable control sequences for use in bacterial host cells include promoters of the *lac* system, the

trp system, the *TAC* or *TRC* system, and the major promoter regions of phage lambda.

                The presence or absence of a signal peptide will, e.g., depend on the expression

host cell used for the production of the polypeptide to be expressed (whether it is an intracellular

30   or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in

filamentous fungi, the signal peptide can conveniently be derived from a gene encoding an

Aspergillus sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or

protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene

encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or

35   *A. niger* glucoamylase. For use in insect cells, the signal peptide can conveniently be derived

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5   from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic

hormone precursor, (cf. US 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid

UDPglucosyltransferase (egt) (Murphy *et al.*, Protein Expression and Purification 4, 349-357

(1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A

preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light

10   chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells

suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf.

US 4,870,008), a modified carboxypeptidase signal peptide (cf. L.A. Valls *et al.*, Cell 48, 1987,

pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3

(YAP3) signal peptide (cf. M. Egel-Mitani *et al.*, Yeast 6, 1990, pp. 127-137), and the synthetic

15   leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been

found to be the signal peptide *ompA* (EP581821).

The nucleotide sequence of the invention encoding a FVII polypeptide, whether

prepared by site-directed mutagenesis, synthesis, PCR or other methods, can optionally include a

nucleotide sequence that encode a signal peptide. The signal peptide is present when the

20   polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, if

present, should be one recognized by the cell chosen for expression of the polypeptide. The

signal peptide can be homologous (e.g., be that normally associated with hFVII) or heterologous

(i.e., originating from another source than hFVII) to the polypeptide or can be homologous or

heterologous to the host cell, i.e., be a signal peptide normally expressed from the host cell or

25   one which is not normally expressed from the host cell. Accordingly, the signal peptide can be

prokaryotic, e.g., derived from a bacterium such as *E. coli,* or eukaryotic, e.g., derived from a

mammalian, or insect or yeast cell.

Any suitable host can be used to produce the polypeptide or polypeptide part of

the conjugate of the invention, including bacteria, fungi (including yeasts), plant, insect,

30   mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants.

Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus,* e.g., *B.*

brevis or *B. subtilis, Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of

E. coli. The introduction of a vector into a bacterial host cell can, for instance, be effected by

protoplast transformation (see, *e.g.,* Chang and Cohen, 1979, *Molecular General Genetics* 168:

35   111-115), using competent cells (see, *e.g.,* Young and Spizizin, 1961, *Journal of Bacteriology*

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5   81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g., *A. oryzae*, *A. niger*, or *A. nidulans, Fusarium* or *Trichoderma*. Fungal cells can be transformed by a process

10   involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g., *S. cerevisiae*,

15   *Schizosaccharomyces, Klyveromyces, Pichia*, such as *P. pastoris* or *P. methanolica, Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast can be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; Hinnen *et al.*, 1978, *Proceedings*

20   *of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (US 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein can be

25   performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g., CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g., COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g., NS/O), Baby Hamster Kidney (BHK) cell lines (e.g., ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g., HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture.

30   Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. Also, the mammalian cell, such as a CHO cell, can be modified to express sialyltransferase, e.g., 1,6-sialyltransferase, e.g., as described in US 5,047,335, in order to provide improved glycosylation of the FVII or FVIIa polypeptide.

5   In order to increase secretion it can be of particular interest to produce the

polypeptide of the invention together with an endoprotease, in particular a PACE (Paired basic

amino acid converting enzyme) (e.g., as described in US 5,986,079), such as a Kex2

endoprotease (e.g., as described in WO 00/28065).

           Methods for introducing exogeneous DNA into mammalian host cells include

10   calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection,

liposome-mediated transfection, viral vectors and the transfection method described by Life

Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the

art and e.g., described by Ausbel *et al.* (eds.), 1996, Current Protocols in Molecular Biology,

John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted

15   according to established methods, e.g., as disclosed in (Animal Cell Biotechnology, Methods and

Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, New Jersey, USA and

Harrison MA and Rae IF, General Techniques of Cell Culture, Cambridge University Press

1997).

           In the production methods of the present invention, the cells are cultivated in a

20   nutrient medium suitable for production of the polypeptide using methods known in the art. For

example, the cell can be cultivated by shake flask cultivation, small-scale or large-scale

fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory

or industrial fermenters performed in a suitable medium and under conditions allowing the

polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient

25   medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in

the art. Suitable media are available from commercial suppliers or can be prepared according to

published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the

polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from

the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

30   The resulting polypeptide can be recovered by methods known in the art. For

example, the polypeptide can be recovered from the nutrient medium by conventional procedures

including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or

precipitation.

           The polypeptides can be purified by a variety of procedures known in the art

35   including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic,

5   chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric

focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, or

extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers,

New York, 1989).

Single chain FVII can be purified and activated to two-chain FVIIa by a number

10   of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47

and Hedner and Kisiel, 1983, J.Clin.Invest. 71:1836-41). Another method whereby single chain

FVII can be purified is by incorporation of Zn ions during purification as described in US

5,700,914.

In a preferred embodiment, the polypeptide is purified as a single chain FVII,

15   which further is PEGylated. The PEGylated FVII single chain polypeptide is activated by either

use of an immobilized enzyme (e.g., factors IIa, IXa, Xa and XIIa) or by autoactivation using a

positively charged ion exchange matrix or the like.

It is advantageous to first purify FVII in its single chain form, then PEGylate (if

desired) and last activate by one of the methods described above or by autoactivation as

20   described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out

PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage

of R152-I153 is avoided. PEGylation of this new amino terminal would render the molecule

inactive since the formation of a hydrogen bond between D242 and the amino terminal of I153 is

necessary for activity.

25   Pharmaceutical composition of the invention and its use
In a further aspect, the present invention relates to a composition, in particular to a

pharmaceutical composition, comprising a polypeptide or conjugate (including an inactive

conjugate as described further above) of the invention and a pharmaceutically acceptable carrier

or excipient.

30   The conjugate, the polypeptide or the pharmaceutical composition according to

the invention can be used as a medicament.

Preferably, the polypeptide or the (active) conjugate can be used for the

manufacture of a medicament for the treatment or prophylaxis of a FVIIa/TF-related disease or

disorder in a mammal. For example, the polypeptide or the (active) conjugate can be used for the

35   manufacture of a medicament for the treatment or prophylaxis of diseases wherein increased clot

5   formation is desirable, such as treatment of patients having diseases resulting in inadequate blood

coagulation in response to damage to blood vessels. In particular, the polypeptide or the (active)

conjugate can be used for the manufacture of a medicament for the treatment of hemophiliacs,

hemophiliacs with inhibitors to FVIII and FIX, patients with thrombocytopenia, patients with

thrombocytopathies, such as Glanzmann's thrombastenia platelet release defects and storage

10   pool defects, patients with von Willebrand's disease, patients with liver diseases, or otherwise

healthy people with severe bleeding problems, e.g., due to trauma or major surgery, who have

developed inhibitors to FVIIa, bleeding disorders such as hemophiliacs and other typically

associated with severe tissue damages.

Analogously, the inactive conjugate of the invention can be used for the

15   manufacture of a medicament for the treatment or prophylaxis of a FVIIa/TF-related disease or

disorder in a mammal. For example, the inactive conjugate of the invention can be used for the

manufacture of a medicament for the treatment or prophylaxis of diseases where decreased clot

formation is desirable, such as prophylaxis or treatment of patients being in hypercoagulable

states, such as patients with sepsis, deep-vein thrombosis, patients in risk of myocardial

20   infections or thrombotic stroke, pulmonary embolism, patients with acute coronary syndromes

(myocardial infarction and unstable angina pectoris), patients undergoing coronary cardiac,

prevention of cardiac events and restonosis for patients receiving angioplasty, patients with

peripheral vascular diseases. The inactive conjugate of the invention can also be used for the

manufacture of a medicament for the treatment of respiratory diseases, tumor growth and

25   metastasis.

In another aspect, the polypeptide, the (active) conjugate or the pharmaceutical

composition comprising the (active) conjugate of the invention can be used in a method for

treating a mammal having a FVIIa/TF-related disease or disorder (such as one or more of the

diseases or disorders mentioned above), comprising administering to a mammal in need thereof

30   an effective amount of such a polypeptide, conjugate or composition.

Analogously, the inactive conjugate or the pharmaceutical composition

comprising the inactive conjugate of the invention can be used in a method for treating a

mammal having a FVIIa/TF-related disease or disorder (such as one or more of the diseases or

disorders mentioned above), comprising administering to a mammal in need thereof an effective

35   amount of such an inactivated conjugate or composition.

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5   The polypeptides or conjugates of the invention is administered to patients in a
therapeutically effective dose, normally one approximately paralleling that employed in therapy
with rFVII such as NovoSeven®, or at higher dosage. By "therapeutically effective dose" herein
is meant a dose that is sufficient to produce the desired effects in relation to the condition for
which it is administered. The exact dose will depend on the circumstances, and will be
10   ascertainable by one skilled in the art using known techniques. Normally, the dose should be
capable of preventing or lessening the severity or spread of the condition or indication being
treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide,
conjugate or composition of the invention depends, inter alia, upon the disease, the dose, the
administration schedule, whether the polypeptide or conjugate or composition is administered
15   alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions,
and the general health of the patient. Preferably, the polypeptide, conjugate, or composition of
the invention is administered in an effective dose, in particular a dose which is sufficient to
normalize the coagulation disorder.

        The polypeptide or conjugate of the invention is preferably administered in a
20   composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically
acceptable" means a carrier or excipient that does not cause any untoward effects in patients to
whom it is administered. Such pharmaceutically acceptable carriers and excipients are well
known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th edition, A. R.
Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of
25   Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000] ; and
Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press
[2000]).

        The polypeptide or conjugate of the invention can be formulated into
pharmaceutical compositions by well-known methods. Suitable formulations are described by
30   Remington's Pharmaceutical Sciences by E.W. Martin (Mark Publ. Co., 16th Ed., 1980).

        The polypeptide or conjugate of the invention can be used "as is" and/or in a salt
form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline
earth metals, such as sodium, potassium, calcium and magnesium, as well as e.g., zinc salts.
These salts or complexes can be present as a crystalline and/or amorphous structure.

5   The pharmaceutical composition of the invention can be administered alone or in

conjunction with other therapeutic agents. These agents can be incorporated as part of the same

pharmaceutical composition or can be administered separately from the polypeptide or conjugate

of the invention, either concurrently or in accordance with another treatment schedule. In

addition, the polypeptide, conjugate or pharmaceutical composition of the invention can be used

10   as an adjuvant to other therapies.

A "patient" for the purposes of the present invention includes both humans and

other mammals. Thus the methods are applicable to both human therapy and veterinary

applications.

The pharmaceutical composition of the polypeptide or conjugate of the invention

15   can be formulated in a variety of forms, e.g., as a liquid, gel, lyophilized, or as a compressed

solid. The preferred form will depend upon the particular indication being treated and will be

apparent to one skilled in the art.

In particular, the pharmaceutical composition of the polypeptide or conjugate of

the invention can be formulated in lyophilised or stable soluble form. The polypeptide or the

20   conjugate can be lyophilised by a variety of procedures known in the art. A polypeptide or the

conjugate can be a stable soluble form by the removal or shielding of proteolytic degradation

sites. The advantage of obtaining a stable soluble preparation lies in easier handling for the

patient and, in the case of emergencies, quicker action, which potentially can become life saving.

The preferred form will depend upon the particular indication being treated and will be apparent

25   to one of skill in the art.

The administration of the formulations of the present invention can be performed

in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously,

intracerebrally, intranasally, transdermally, intraperitoneally, intramuscularly, intrapulmonary,

vaginally, rectally, intraocularly, or in any other acceptable manner. The formulations can be

30   administered continuously by infusion, although bolus injection is acceptable, using techniques

well known in the art, such as pumps or implantation. In some instances the formulations can be

directly applied as a solution or spray.

Parenterals
A preferred example of a pharmaceutical composition is a solution designed for

35   parenteral administration. Although in many cases pharmaceutical solution formulations are

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5   provided in liquid form, appropriate for immediate use, such parenteral formulations can also be

provided in frozen or in lyophilized form. In the former case, the composition must be thawed

prior to use. The latter form is often used to enhance the stability of the active compound

contained in the composition under a wider variety of storage conditions, as it is recognized by

those skilled in the art that lyophilized preparations are generally more stable than their liquid

10   counterparts. Such lyophilized preparations are reconstituted prior to use by the addition of one

or more suitable pharmaceutically acceptable diluents such as sterile water for injection or sterile

physiological saline solution.

In case of parenterals, they are prepared for storage as lyophilized formulations or

aqueous solutions by mixing, as appropriate, the polypeptide having the desired degree of purity

15   with one or more pharmaceutically acceptable carriers, excipients or stabilizers typically

employed in the art (all of which are termed "excipients"), for example buffering agents,

stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents, antioxidants

and/or other miscellaneous additives.

Buffering agents help to maintain the pH in the range which approximates

20   physiological conditions. They are typically present at a concentration ranging from about 2 mM

to about 50 mM Suitable buffering agents for use with the present invention include both organic

and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium

citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture,

etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium

25   hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric

acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium

hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture,

fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture,

etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium

30   hydroxide mixture, gluconic acid-potassium glyuconate mixture, etc.), oxalate buffer (e.g., oxalic

acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium

oxalate mixture, etc.), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium

hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic

acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additional

35   possibilities are phosphate buffers, histidine buffers and trimethylamine salts such as Tris.

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Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose, stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur-containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thiosulfate; low molecular weight polypeptides (i.e., <10 residues); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; monosaccharides such as xylose, mannose, fructose and glucose; disaccharides such as lactose, maltose and sucrose; trisaccharides such as raffinose, and polysaccharides such as dextran. Stabilizers are typically present in the range of from 0.1 to 10,000 parts by weight based on the active protein weight.

Preservatives are added to retard microbial growth, and are typically added in amounts of about 0.2%-1% (w/v). Suitable preservatives for use with the present invention include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalkonium halides (e.g., benzalkonium chloride, bromide or iodide), hexamethonium chloride, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol and 3-pentanol.

Isotonicifiers are added to ensure isotonicity of liquid compositions and include polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Polyhydric alcohols can be present in an amount between 0.1% and 25% by weight, typically 1% to 5%, taking into account the relative amounts of the other ingredients.

Non-ionic surfactants or detergents (also known as "wetting agents") can be present to help solubilizing the therapeutic agent as well as to protect the therapeutic polypeptide against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stress without causing denaturation of the polypeptide. Suitable non-ionic surfactants

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5   include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic® polyols,

polyoxyethylene sorbitan monoethers (Tween®-20, Tween®-80, etc.).

Additional miscellaneous excipients include bulking agents or fillers (e.g., starch),

chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E) and

cosolvents.

10   The active ingredient can also be entrapped in microcapsules prepared, for

example, by coascervation techniques or by interfacial polymerization, for example

hydroxymethylcellulose, gelatin or poly-(methylmethacylate) microcapsules, in colloidal drug

delivery systems (for example liposomes, albumin microspheres, microemulsions, nano-particles

and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's

15   Pharmaceutical Sciences, *supra*.

Parenteral formulations to be used for *in vivo* administration must be sterile. This

is readily accomplished, for example, by filtration through sterile filtration membranes.

Sustained release preparations

Examples of sustained-release preparations include semi-permeable matrices of

20   solid hydrophobic polymers containing the polypeptide or conjugate, the matrices having a

suitable form such as a film or microcapsules. Examples of sustained-release matrices include

polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) or poly(vinylalcohol)),

polylactides, copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-

vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the ProLease® technology

25   or Lupron Depot® (injectable microspheres composed of lactic acid-glycolic acid copolymer and

leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-

vinyl acetate and lactic acid-glycolic acid enable release of molecules for long periods such as up

to or over 100 days, certain hydrogels release proteins for shorter time periods. When

encapsulated polypeptides remain in the body for a long time, they can denature or aggregate as

30   a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible

changes in immunogenicity. Rational strategies can be devised for stabilization depending on the

mechanism involved. For example, if the aggregation mechanism is discovered to be

intermolecular S-S bond formation through thio-disulfide interchange, stabilization can be

achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling

60

5   moisture content, using appropriate additives, and developing specific polymer matrix

compositions.

The invention is further described in the following non-limiting examples.

MATERIALS AND METHODS

Methods used to determine the amino acids to be modified

10   *Accessible Surface Area (ASA)*

The computer program Access (B. Lee and F.M.Richards, J. Mol.Biol. 55: 379-400 (1971)) version 2 (© 1983 Yale University) are used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4Å and defines the Accessible Surface Area (ASA) as the area formed by the center of the probe.

15   Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of side chain

The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms

20   of that residue type in an extended Ala-x-Ala tripeptide (See Hubbard, Campbell & Thornton (1991) J.Mol.Biol.220,507-530). For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table is used as standard 100% ASA for the side chain:

Ala	69.23 Å^2		Leu	140.76 Å^2
Arg	200.35 Å^2		Lys	162.50 Å^2
Asn	106.25 Å^2		Met	156.08 Å^2
Asp	102.06 Å^2		Phe	163.90 Å^2
Cys	96.69 Å^2		Pro	119.65 Å^2
Gln	140.58 Å^2		Ser	78.16 Å^2
Glu	134.61 Å^2		Thr	101.67 Å^2
Gly	32.28 Å^2		Trp	210.89 Å^2
His	147.00 Å^2		Tyr	176.61 Å^2
Ile	137.91 Å^2		Val	114.14 Å^2

5

        Residues not detected in the structure are defined as having 100% exposure as

they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6,

7, 14, 16, 19, 20, 25, 26, 29 and 35 are all defined as being 100% exposed.

Determining distances between atoms

10

        The distance between atoms is most easily determined using molecular graphics

software e.g., InsightII® v. 98.0, MSI INC.

Catalytic site region

        The catalytic site region is defined as any residues having at least one atom within

10 Å of any atom in the catalytic triad (residues H193, D242, S344).

15   *Determination of tissue factor binding site*

        The receptor-binding site is defined as comprising of all residues having their

accessible surface area changed upon receptor binding. This is determined by at least two ASA

calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the

complete ligand(s)/receptor(s) complex.

20   ## Methods for testing FVII and FVIIa properties

Measurement of functional in vivo half-life

        Measurement of *in vivo* biological half-life can be carried out in a number of ways

as described in the literature. An example of an assay for the measurement of *in vivo* half-life of

rFVIIa or variants thereof is described in FDA reference number 96-0597. Briefly, FVII clotting

25   activities are measured in plasma drawn prior to and during a 24-hour period after administration

of the conjugate, polypeptide or composition. The median apparent volume of distribution at

steady state is measured and the median clearance determined.

        ## Measurement of reduced sensitivity to proteolytic degradation

        A composition containing the conjugate (100-750 µg/ml, preferably 600 µg/ml),

30   1.5 mg Ca^{2+}/ml (as calcium chloride), mannitol (30 mg/ml), polysorbate 80 (0.1 mg/ml), sodium

chloride (3 mg/ml) and glycyl-glycine buffer (1.3 mg/ml, pH 5.5) is prepared.

        A similar composition containing wild-type rFVIIa is prepared.

        The initial clotting activity or, alternatively, the initial amidolytic activity is then

determined as described in the section entitled *"Method for measuring the clotting activity"* or as

5   described in the section entitled *"Method of measuring low levels of catalytic activity"* or *"Method of measuring the catalytic activity."*

The compositions are then incubated at 37°C until the composition containing the wild-type rFVII has lost at least 25%, preferably at least 50%, of its initial clotting or amidolytic acitivity.

10   The clotting or amidolytic activity of the composition containing the conjugate of the invention is then measured.

The reduced sensitivity to proteolytic degradation of the conjugate of the invention as compared to wild-type rFVIIa is then expressed in percentage.

Alternative methods for measuring reduced sensitivity to proteolytic degradation
15   Proteolytic degradation can be measured using the assay described in US 5,580,560, Example 5, where proteolysis is autoproteolysis.

Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of wild type and conjugates by withdrawing blood samples and subjecting these to SDS-PAGE and autoradiography.

20   Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to that obtained by non-conjugated wild type FVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels, HPLC or as measured by conserved catalytic activity in comparison to wild type using the chromogenic assay described below.

25   *Determination of the molecular weight of rFVII and conjugates thereof*

The molecular weight of conjugated or unconjugated rFVII or conjugates thereof is determined by either SDS-PAGE, gel filtration, Western Blots, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation, e.g., SDS-PAGE according to Laemmli, U.K., Nature Vol 227 (1970), pp. 680-85.

30   *Method of measuring low levels of catalytic activity*

The amidolytic activity in dilute samples of FVII/FVIIa and fermentation liquid/conditioned medium can be determined using COASET® FVII (Chromogenix, Art. No 82 19 00). The amidolytic activity is determined in accordance with the manufacturer's instructions. Briefly, FX is present in surplus and converted to FXa by FVIIa at 37°C. The generated FXa then
35   hydrolyses the chromogenic substrate S2765 (N-α-Cbo-D-Arg-Gly-Arg-pNA) resulting in

5　　liberation of the chromophoric molecule, para-nitro-anillin (pNA) absorbing light at wavelength

405 nm. The reaction is stopped by addition of acetic acid. The amount of FVII/FVIIa in the

sample is determined by comparison to a standard curve prepared from FVIIa (ranging from 125

pg/ml to 1 ng/ml in assay buffer).

Method of measuring the catalytic activity

10　　　　　　The ability of the conjugates to cleave small peptide substrates can be measured

using the chromogenic substrate S-2288 (D-Ile-Pro-Arg-p-nitroanilide). Recombinant FVIIa is

diluted in 0.1 M Tris, 0.1 M NaCl, 5 mM $CaCl_2$, pH 8.3 containing 0.1% BSA. The reaction is

started by addition of the S-2288 substrate to 1 mM and the absorption at 405 nm is measured

after incubation for 30 min. at 37°C.

15　*Method of measuring the clotting activity*

　　　　　　FVIIa activity is measured using a standard one-stage clotting assay essentially as

described in WO92/15686. Briefly, the sample to be tested is diluted in 50 mM Tris (pH 7.5),

0.1% BSA and 100 μl is incubated with 100 μl of FVII deficient plasma and 200 μl of

thromboplastin C containing 10 mM Ca^{++}. Clotting times are measured and compared to a

20　standard curve using a pool of citrated normal human plasma in serial dilution.

Method of measuring the anticoagulant activity

　　　　　　The anticoagulant activity of an inactive FVII or FVIIa conjugate can be

measured using the one-stage clotting assay described above (*Method of measuring the clotting

activity*) where the inactive conjugate competes with wild-type FVII for a limited amount of

25　relipidated tissue factor. The assay is performed essentially as described in WO 92/15686,

example III, which is hereby incorporated as reference. The ability of the inactive conjugate to

prolong the clotting time of wild-type FVII is recorded and taken as a measure of anticoagulant

activity.

EXAMPLES

30　<u>EXAMPLE 1</u>

　　　　　　The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner

et.al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of

residues in the reference does not follow the sequence. Here we have used the sequential

numbering according to SEQ ID NO:1. The gamma-carboxy glutamic acids at positions 6, 7, 14,

5   16, 19, 20, 25, 26, 29 and 35 are all here named GLU (three letter abbreviation) or E (one letter
     abbreviation). Residues 143-152 are not present in the structure.

Surface exposure

Performing fractional ASA calculations on FVII fragments alone combined with
the definition of accessibilities of non standard and/or missing residues described in the methods

10   resulted in the following residues having more than 25% of their side chain exposed to the
     surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21,
     S23, F24, E25, E26, R28, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45,
     G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73,
     P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97,

15   E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121,
     A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143,
     R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161,
     E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203,
     I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237,

20   T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271,
     F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296,
     N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320,
     P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353,
     G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395,

25   R396, P397, G398, V399, L400, L401, R402, P404 and P406.

The following residues had more than 50% of their side chain exposed to the
surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26,
E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62,
L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89,

30   I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123,
     G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151,
     R152, G155, K157, P160, N173, G174, A175, K197, K199, N200, R202, S214, E215, H216,
     G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278,
     L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313,

5   R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392,

E394, R396, P397, G398, R402, P404 and P406.

Tissue factor binding site

Performing ASA calculations the following residues in human FVII change their

ASA in the complex. These residues were defined as constituting the receptor binding site: L13,

10   K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73,

P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277,

F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325 and R379.

Active site region

The active site region is defined as any residue having at least one atom within a

15   distance of 10 Å from any atom in the catalytic triad (residues H193, D242, S344): I153, Q167,

V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191,

A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234,

V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282,

G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341,

20   G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364,

G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400 and F405.

The ridge of the active site binding cleft

The ridge of the active site binding cleft region was defined by visual inspection

of the FVIIa structure 1FAK.pdb as: N173, A175, K199, N200, N203, D289, R290, G291, A292,

25   P321 and T370.

EXAMPLE 2

Design of an expression cassette for expression of human blood coagulation factor VII in
mammalian cells

The DNA sequence shown in SEQ ID NO:2, encompassing the short form of the

30   full length cDNA encoding human blood coagulation factor VII with its native short signal

peptide (Hagen et al., 1986. PNAS 83:2412), was synthesized in order to facilitate high

expression in mammalian cells. First the ATG start codon context was modified according to the

Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50), so that there is a

perfect match to the consensus sequence upstream of the ATG start codon. Secondly the open

35   reading frame of the native human blood coagulation factor cDNA was modified by making a

66

5   bias in the codon usage towards the codons frequently used in highly expressed human genes. Further, two translational stop codons was inserted at the end of the open reading frame in order to facilitate efficient translational stop. The fully synthetic and expression optimized human FVII gene was assembled from 70-mer DNA oligonucleotides and finally amplified using end primers inserting *Bam*HI and *Hind*III sites at the 5' and 3' ends respectively using standard PCR

10   techniques, which resulted in the following sequence (SEQ ID NO:4):

ggatcccgccaccatggtcagccaggccctccgcctcctgtgcctgctcctgggggctgcagggctgcctggctgccgtcttcgtcacccag

gaggaagcccatggcgtcctgcatcgccggcgccgggccaatgcctttctggaagagctccgccctggctccctggaacgcgaatgcaa

agaggaacagtgcagctttgaggaagcccgggagattttcaaagacgctgagcggaccaaactgttttggattagctatagcgatggcgat

cagtgcgcctccagcccttgccagaacggggggctcctgcaaagaccagctgcagagctatatctgcttctgcctgcctgcctttgaggggc

15   gcaattgcgaaacccataaggatgaccagctgatttgcgtcaacgaaaacggggctgcgagcagtactgcagcgatcacacgggcacg

aagcggagctgccgctgccacgaaggctatagcctcctggctgacggggtgtcctgcacgcccacggtggaatacccttgcgggaagatt

cccattctagaaaagcggaacgctagcaaaccccagggccggatcgtcggcgggaaggtctgccctaagggggagtgcccctggcagg

tcctgctcctggtcaacggggcccagctgtgcggcgggaccctcatcaataccatttgggtcgtgtccgccgctcactgcttcgataagatta

agaattggcggaacctcatcgctgtgctcggcgaacacgatctgtccgagcatgacgggggacgaacagtcccgccgggtggctcaggtc

20   atcattccctccacctatgtgcctggcacgaccaatcacgatatcgctctgctccgcctccaccagcccgtcgtgctcaccgatcacgtcgtg

cctctgtgcctgcctgagcggacctttagcgaacgcacgctggctttcgtccgctttagcctcgtgtccggctggggccagctgctcgaccg

gggcgctaccgctctcgagctgatggtgctcaacgtcccccggctgatgacccaggactgcctgcagcagtcccgcaaagtgggggact

cccccaatatcacggagtatatgttttgcgctggctatagcgatggctccaaggatagctgcaaggggggactccggcgggccccatgccac

gcactatcgcgggacctggtacctcaccgggatcgtcagctggggccagggctgcgccacggtggggcactttggcgtctacacgcgcg

25   tcagccagtacattgagtggctgcagaagctcatgcggagcgaaccccggcccggggtgctcctgcgggcccctttcccttgataaaagct

t

A vector for the cloning of the generated PCR product encompassing the expression cassette for native human blood coagulation factor VII was prepared by cloning the intron from pCINeo (Promega). The synthetic intron from pCI-Neo was amplified using standard

30   PCR conditions as described above and the primers:

CBProFpr174: 5'- AGCTGGCTAGCCACTGGGCAGGTAAGTATCA -3' and
CBProFpr175: 5'- TGGCGGGATCCTTAAGAGCTGTAATTGAACT -3'

resulting in a 332 bp PCR fragment. The fragment was cut with *Nhe*I and *Bam*HI before cloning into pCDNA3.1/HygR (obtained from In Vitro Gen) resulting in PF#34.

5   The expression cassette for native human blood coagulation factor VII was cloned

between the *Bam*HI and *Hind*III sites of PF#34, resulting in plasmid PF#226.

EXAMPLE 3

Construction of expression cassettes encoding variant forms of human blood coagulation factor

VII that are having an additional in vivo glycosylation site.

10   Sequence overhang extension (SOE) PCR was used for generating constructs

having variant human blood coagulation factor open reading frames with substituted codons. In

the SOE-PCR both the N-terminal part and the C-terminal part of the FVII open reading frame

was first amplified in individual primary PCRs.

          For example, in order to change the codons for R315 and V317 to the codons for

15   N315 and T317 the following primers were used pair vice for the primary PCR's:

CBProFpr216: 5'-CTTAA<u>GGATCC</u>CGCCACCATGGTCAGCCAG-3' and

CBProFpr229: 5'-GGAGTCCCCGGTTTTGTTGGACTGCTGC-3',

and

CBProFpr221: 5'-ACTT<u>AAGCTT</u>TTATCAAGGGA-3' and

20   CBProFpr228: 5'-GCAGCAGTCCAACAAAACCGGGGACTCC-3'.

          The primary PCR products were then combined and the terminal primers

(CBProFpr216 and CBProFpr221) added resulting in the secondary full-length product encoding

the desired R315N + V317T FVII variant. The secondary PCR product was trimmed with

BamHI and HindIII before cloning into the vector PF#34 between the *Bam*HI and *Hind*III sites

25   resulting in PF#249.

          Furthermore, in cases where the introduced mutation(s) were sufficiently close to

a unique restriction endo-nuclease site in the expression plasmid variant genes were constructed

using construction procedure encompassing a single PCR step and a subsequent cloning. For

instance, the substitution K143N+N145T was introduced by use of the PCR primers:

30   CBProFpr226: 5'-CATTCTAGAAAACCGGACCGCTAGCAAACC-3' and

CBProFpr221: 5'- ACTTAAGCTTTTATCAAGGGA-3'

in a single PCR reaction.

          The PCR product was subsequently cloned using the restriction endo-nuclease

sites *Xba*I and *Hind*III.

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5   Using the above strategy, the following glycosylation conjugates were prepared

and their amidolytic activities were tested as described in the section entitled *Method of*

measuring low levels of catalytic activity. Furthermore, some of the conjugates were subjected to

the one-stage clotting assay described in the section entitled *Method of measuring the clotting*

activity. The obtained results are compiled in the following table.

Glycosylation Conjugate	Amidolytic activity	Clotting activity
T106N	+	+
K143N + N145T	+	nd
V253N	+	nd
R290N + A292T	-	nd
G291N	-	nd
R315N + V317T	+	+
K143N + N145T + R315N + V317T	+	nd

10   +: Activity measurable; -: Activity not measurable; nd: not determined

EXAMPLE 4

Improvement of glycosylation site utility by introduction of another proximal (N-terminally

located) glycosylation site.

          In order to prevent autolysis of wild type human FVII a glycosylation site was

15   introduced at position 315 by making the substitutions: R315N and V317T as described above,

resulting in PF#249.

          Upon transfection of CHO K1 cells, using Lipofactamine 2000, low transient

expression levels were obtained. Assaying the twenty-four hour transient supernatant by the

amidiolytic assay, COASET® FVII, indicated that the variant was active. After selection using

20   400 μg/ml Hygromycin B a pool of stable clones was obtained. This pool expresses the

R315N+V317T variant at approximately 0.2 μg/ml allowing for Western blotting analysis for

determination of the degree of usage of the introduced glycosylation site. A twenty-four hour

supernatant from the stable pool was assayed by Western blotting and revealed partial usage of

the introduced glycosylation site at position 315, approximately one-half of the fully processed

25   secreted protein is glycosylated. However, if the native glycosylation site at position 145 is

moved to position 143, by making the substitutions K143N+N145T, the introduced glycosylation

site at position 315 is completely glycosylated as judged by Western blotting.

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EXAMPLE 5

Expression of FVII in HEK 293 cells

The cell line HEK 293 (ATCC # CRL-1573) was seeded at 20% confluence in T-25 flasks using DMEM, high glucose 10% heat inactivated FCS (Gibco/BRL Cat # 10091), 5μg/ml phylloquinone and allowed to grow until confluent. The confluent mono cell layer was transfected with 1, 2, 5, 10 and 20 μg of the plasmid p226 described above using the Lipofectamine 2000 transfection agent (Life technologies) according to the manufactures instructions. After 24 hours post transfection a medium sample was drawn. The FVII concentration in the 24 hour transient expression experiments was on average 0.15 μg/ml.

Subsequently, selection medium containing 100 μg/ml Hygromycin B was administered to the cells. After three weeks of selection, with renewal of the medium every 3rd or 4th day, the Hygromycin resistant cells were confluent in the flasks transfected with 1μg of plasmid DNA and 2μg of plasmid DNA. The cells from each of the five flasks were harvested and pooled. The resulting stable pool of transfectants expressing native human blood coagulation factor VII was frozen in liquid nitrogen according to standard procedures.

EXAMPLE 6

Generation of HEK293 cells stably expressing FVII.

A vial of HEK293 PF#226 transfectant pool was thawed and the cells seeded in a 75 cm^2 tissue flask containing 15 ml of DMEM high glucose, 10% FCS, phylloquinone (5 μg/ml), 100 U/l penicillin, 100μg/l streptomycin, which was used for all subsequent experiments, and grown for 24 hours. The cells were harvested, diluted and plated in 96 well micro titer plates at a cell density of ½ cell/well. After 12 days colonies of about 20-100 cells were present in the wells and those wells containing only one colony were labeled. After a further two days of growth an additional 100 μl medium was added to all wells. Two days later the media in all wells containing only one colony were changed. The first colonies were transferred to 25 cm^2 tissue flasks culturing after 3 days and depending on the level of confluency the colonies were transferred to 25 cm^2 tissue flasks culturing the next 11 days. When confluent in T-25 tissue flasks the medium was changed and clones allowed to secrete FVII into the growth medium for 24 hours, after which the supernatants were harvested and assayed for the presence of factor VII using the COASET FVII amidolytic assay. One clone, C18, was found to express 29 μg/ml FVII.

EXAMPLE 7

Expression of FVII glycosylation variants with no amidolytic activity capable of inhibiting the function of FVIIa

The expression plasmids for expression of the active site ridge mutants R290N+A292T and G291N were constructed essentially as described in example 3.

Using the amidiolytic assay COASET® FVII (see above) the ability of the two FVII glycosylation variants, R290N+A292T and G291N, to inhibit the activity of rFVIIa was evaluated. The plasmids PF#250 encoding R290N+A292T and the plasmid PF#294 encoding G291N were transfected into near confluent serum grown HEK293 cells using Lipofectamin 2000. The transfected cells were incubated at 37°C with 5% CO_2 for three hours post transfection before the medium was changed to serum free medium (DMEM, ITS-A, ExCyte, Phylloquinone, P/S). Forty hours post transfection the conditioned medium was harvested and cleared by centrifugation for analysis.

A standard curve was made from rFVIIa: 0.0125 ng, 0.025 ng, 0.05 ng, 0.075 ng and 0.1 ng. Fifty μl aliquots of undiluted, 2-fold diluted, 5 fold diluted, 10-fold diluted and 50-fold diluted condition medium from either of the two inactive glycosylation variants R290N+A292T and G291N or conditioned medium from a mock transfection were spiked with 0.025 ng rFVIIa. When assayed in the COASET FVII assay 0.025 ng of FVIIa corresponded to a signal of OD_{405} = 0.35 (first run) and OD_{405} = 0.26 (second run).

The obtained results are compiled in the following table:

Glycosylation Conjugate	Dilution	OD_{405}	
Standard		0.35	0.26
Mock	50	0.38	0.19
	25	0.31	0.16
	10	0.21	0.15
	5	0.22	0.14
	1	0.08	0.07
R290N + A292T	50	0.23	-
	25	0.12	-
	10	0.07	-
	5	0.04	-
	1	0.04	-
G291N	50	-	0.16
	25	-	0.08
	10	-	0.06
	5	-	0.05
	1	-	0.04

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5   As it appears from the above data the glycosylation conjugates G291N and

R290N+A292T inhibit the function rFVIIa.

EXAMPLE 8

Purification of FVII and Subsequent Activation

Purification of wild-type Factor VII and conjugates thereof was performed at 4°C.

10   The supernatant from cells expressing the conjugate (or wild-type FVII) was sterile filtered (0.22

μm) and diluted 2 fold in cold milliQ water. EDTA was added to 5 mM, pH adjusted to 8.6 and

conductivity was below 10 mS/cm. The sample was applied onto a Q-sepharose Fast Flow resin

(Pharmacia) equilibrated at 4°C in 10 mM Tris pH 8.6. After application of the sample the

column was washed in 10 mM Tris (pH 8.6), 150 mM NaCl, until the absorption at 280 nm

15   reached baseline levels. Then the column was equilibrated in 10 mM Tris (pH 8.6), 100 mM

NaCl. Bound conjugate (or wild-type FVII) was eluted with 10 mM Tris (pH 8.6), 100 mM

NaCl, 5 mM CaCl$_2$. Fractions enriched in conjugate (or wild-type FVII) was pooled and

concentrated by dialysis or by using Vivaspin concentration units (Vivascience).

Auto-activation of the conjugate (or wild-type FVII) was obtained by

20   concentration and incubation of the eluted protein in 10 mM Tris (pH 7.8-8.6), 100 mM NaCl, 5

mM CaCl$_2$.

Alternatively, the conjugate (or wild-type FVII) was activated at 37°C by Factor

Xa coupled to CNBr-activated sepharose in 10 mM Tris (pH 7.4-8.0) 100 mM NaCl, 5 mM

CaCl$_2$.

25   The conjugate (or wild-type FVIIa) was buffer-exchanged into a solution

containing 10 mM CaCl$_2$, 50 mM NaCl, 3% mannitol, 0.05% Tween80, buffered at pH 5.6 and

sterilfiltered before storage at -80°C.

EXAMPLE 9

N-Terminal Pegylation of FVII

30   Factor VII was conjugated with methoxy polyethylene glycol (mPEG) having a

molecular weight of about 5 kDa using M-PEG-CHO (M-ALD-5000, obtained from Shearwater)

in a buffer containing 10 mM sodium citrate, 20 mM CaCl$_2$, 100 mM NaCl, pH 5.5. M-PEG-

CHO was present in 50-100-fold molar excess, and the protein concentration was 0.2-0.5 mg/ml.

The reaction was carried out in 300-1500 μl batches at room temperature for 1 hour with

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5   agitation, and NaBH₃CN was added to 500-1000 fold excess and incubation continued over night with agitation at room temperature.

PEGylated FVII was buffer exchanged to buffer A (10 mM Tris pH 7.6) and applied at 4 °C on a mono Q column (Pharmacia) equilibrated in buffer A. Bound protein was eluted in a gradient from 0-100% B (10 mM Tris (pH 7.6), 500 mM NaCl) over 40 column

10   volumes.

EXAMPLE 10

Pharmakokinetic Studies in Rats

Both wild-type FVII and the conjugates of the invention are formulated in 1.3 mg/ml glycyl-glycin buffer pH 5.5 containing 1.5 mg/ml $CaCl_2$, 30 mg/ml mannitol, 0.1 mg/ml

15   polysorbat 80 and 3 mg/ml NaCl. For determination of *in vivo* half-life each of the preparations are administered to Sprague-Dawley rats as one intra venous bolus injection. The injections are given slowly over about 10 seconds to reduce potential risk of heart failure due to high Ca^{2+} concentration. Blood samples are drawn from each of the nine anaesthetized rats at suitable intervals, e.g., 1 minute, 15 minutes, 30 minutes, 45 minutes and 1 hour after injection. The

20   blood samples are collected in 1 ml tubes containing 50 μl Citrate-phosphate-dextrose solution with adenine (Sigma #C4431) in order to prevent coagulation. Immediately after collection the samples are stored at about 0°C until centrifugation and subsequent collection of the citrate plasma supernatants for assay. Samples are assayed by the one-stage clotting assay as described in the section *Method of measuring the clotting activity* and the half-lives are then calculated.

25   While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above can be used in various combinations. All publications, patents, patent

30   applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, or other document were individually indicated to be incorporated by reference for all purposes.

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